DISSERTATION

SURVEILLANCE AND DIAGNOSIS OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN THE UNITED STATES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MICHELLE MARIE DENNIS ENTITLED SURVEILLANCE AND DIAGNOSIS OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN THE UNITED STATES FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

SURVEILLANCE AND DIAGNOSIS OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN THE UNITED STATES

Since limited knowledge of transmissible spongiform encephalopathies (TSEs) restricts treatment and successful control interventions, and since some may cause fatal food-borne disease in humans, the United States (U.S.) has established TSE surveillance programs to support control efforts and to protect agriculture-based economy. The enhanced BSE surveillance system was conducted to characterize the extent of the presence of BSE in the U.S. cattle population in order to reassure consumers and trading partners of the U.S. BSE status. Given the level of importance and the cost of the enhanced BSE surveillance program, surveillance system evaluation was conducted to provide feedback for improving future surveillance and to determine the extent to which the system had met its objectives. Recommendations were made to improve efficiency and quality of future BSE surveillance systems. The enhanced BSE surveillance certainly met its stated objectives.

Surveillance interests in the U.S. were subsequently re-directed towards efficiently assuring that BSE control measures remain effective, and to maintain assurance of trading partners of the U.S. BSE status. A plan for ongoing BSE surveillance was constructed using the standards and guidelines for animal health surveillance established by the National Surveillance Unit (NSU). Results derived from the enhanced BSE surveillance system and its evaluation prompted appriopriate adaptations for maintenance surveillance methods.

Conditions which naturally degrade prions need to be elucidated to facilitate disposal of prion-contaminated biowastes. In order to determine whether long-term heating could destroy prions, the immunodetection of protease-resistant, disease-associated prion protein (PrP^{res}) was evaluated in brain from chronic wasting disease (CWD)-affected elk. Using 3 diagnostic assays for CWD, progressive loss of PrP^{res} immunodetectability, which increased with incubation temperature, was demonstrated when brain homogenates were incubated at 37, 55, and 80° C over a period of 200 days. Disposal systems which use heat over time may effectively degrade prions. Furthermore, the validity of test results derived from tissues which have been exposed to such conditions is questionable.

In the U.S., scrapie surveillance uses PrP^{res} immunohistochemistry (IHC) applied to tissues collected postmortem. The only live animal test available, PrP^{res} IHC applied to third eyelid biopsy, is limited by comparatively lower sensitivity, high frequency of inconclusive test results, and the limited amount of tissue available for repeat testing. A study evaluated PrP^{res} IHC applied to recto-anal mucosa associated lymphoid tissue (RAMALT) biopsy for scrapie diagnosis in live sheep. Biopsy-related complications were rare. The sensitivity of RAMALT biopsy PrP^{res} IHC ranged from 87.5-89.3%, and approximated or exceeding that applied to third eyelid biopsy. The use of PrP^{res} IHC applied to RAMALT biopsies for scrapie diagnosis in live high-risk sheep is expected to improve the surveillance activities that support the success of the U.S. National Scrapie Eradication Program.

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Since entering the field of veterinary medicine it has been a goal of mine to obtain a PhD. I have regarded this achievement as the ultimate means to substantiate myself as a veterinary scientist. While stimulating and exciting, this task was the most challenging phase of my education and I am grateful beyond words to those who supported me and encouraged my success. I have been privileged that many of these persons are exceptional among their fields of interest. Moreover, these persons are of exceptional character, demonstrating success through generosity, diligence, selflessness, and sincere concern for the greater good of man and animals. It has been a truly overwhelming experience to be trained, looked after, and endorsed by those whom I hold the highest respect for. I aspire to adopt and reflect the nature of these individuals throughout my career and positively influence others as they have me.

Dr. Salman has a gift of identifying and foreseeing topics that need to be addressed by veterinary research. He is open-minded and able to recognize the combinations of training, research team members, and research methods that may bring about success. These characteristics, combined with his magnetic personality and vast network of friends, are in part the basis for his astonishingly productive research track-record. He is a great teacher of our time; one does not need to read about the excess of awards he has received to recognize this, but only witness the degree of fulfillment he gains from the success of his trainees. All of these traits have been more than a little inspirational to me. He has taught me things about research funding, population-based research, international perspective, and professional relationships that I might have never learned with years of

experience. When first meeting Dr. Salman, the impression I got was of a very passionate yet over-worked and busy man, saturated with other commitments and responsibilities, who was willing and excited to sacrifice more of his time and funding to train a veterinarian resident he just met. Now I know that impression reflected only the surface of his generosity. I have been very fortunate to have a mentor who shared the excitement of the potential of combining training in pathology and epidemiology, and had the kindness in his heart to do everything he could to make me successful within and beyond my graduate program. I am blessed to be a member of the cohort trained and befriended by a legendary veterinarian.

Dr. Gould also shared enthusiasm for a training program that would combine pathology and epidemiology; having his support helped prevent conflicts occurring between responsibilities related to my graduate program and residency program. As a teacher, he challenged my scientific thought process more than any other. He had a magical way of helping me recognize mistakes, learn from mistakes, and address limitations without making me feel less intelligent. As a mentor, his door was always open to help alleviate my latest frustrations, and the manner in which he checked up on the progression of my program ensured me that I was going to make it through. Through our discussions about diagnostic case work, research projects, and political and socioeconomic dilemmas of the day, I have grown tremendously and gained perspectives I would not have found on my own. I thank him for helping me grasp the importance of my role in society as a veterinarian and as an educated individual. I have wondered how many great minds he has knowingly and unknowingly developed and where they will surface.

Dr. McCluskey is another well-known and tremendously busy individual who sacrificed his time to help me be successful. I exceedingly respect the savvy and easy-going nature he holds in a challenging career-path. I was so honored to have his trust when tasked with the BSE-related topics of this dissertation. I was even more honored when he made me feel so welcomed to the NSU. I have never been able and still struggle to find the words to thank him properly for using my meek research proposal to acquire 2 years of non-traditional funding for my program. Doing so made such a tremendous difference in my program and in my life that I hope one day I can return the favor to him and to others whom I mentor.

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Several persons who were not members of my committee have had profound impacts on the success of my program. Joni Triantis was the best mentor and friend a student could have in the laboratory. She was passionate about the project we worked on together and

the quality of our results was in a large part due to her expertise. The NSU staff I worked with, including Tracey Lynn, Aaron Scott, Eric Ebel, and Chuck Fossler, were incredibly welcoming to me even though initially I was quite out of my element among their group and more than a little intimidated by their epidemiology knowledge and skills. They were all very patient with my student status and were fun to work with and learn from. I am grateful to Katherine Marshall for thinking of and recommending me when the potential of conducting the scrapie rectal biopsy study first surfaced. I was longing to include a project like this in my dissertation and was so honored to play such an important part in this nationally-executed study. She was also fun to work with and I couldn't have done any of that work without her. Bruce Thompsen contributed sizeable time and effort to conducting the rectal biopsy study and remains very invested in its outcomes. I have deep appreciation for his kindness in giving me a lead role and working so well with me while tolerating my quirks related to being a graduate student. Since the beginning of my PhD work, Bruce Wagner has been an invaluable resource for solving statistical quandaries. He has always been willing to spend time with me to discuss such problems and it has been a privilege to learn through experience from his thought process.

It has been humbling to rub elbows with so many great scientists and professionals during my training. I hope the character of at least some of them have rubbed off on me so that I may influence others as they have me. And I hope during my future career-related endeavors I will find myself surrounded by half as many positive and inspiring people.

PREFACE

The following provides an overview of the structure of this dissertation. The broad theme of the dissertation involves methods and approaches for diagnosis and surveillance of transmissible spongiform encephalopathies (TSEs) which affect domestic animals in the United States.

Chapter 1 is a literature review which presents general concepts of animal disease surveillance systems and examines the history of and methods for transmissible spongiform encephalopathy surveillance adopted by the United States for bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie.

Chapter 2 is a report produced for United States Department of Agriculture (USDA) policy makers and for personnel of the USDA's National Surveillance Unit (NSU). It is a systematic assessment of the U.S. Enhanced BSE Surveillance Program. This study was conducted to test a novel protocol for evaluating animal health surveillance systems and to identify areas for improving future BSE surveillance methods. This chapter is formatted in accordance with the design of the NSU's draft *Protocol for Evaluation of Animal Health Surveillance Systems.*

Chapter 3 is a paper that proposes methods for continued BSE surveillance in the U.S. following the conclusion of the U.S. Enhanced BSE Surveillance Program. This plan was produced in conjunction with the NSU for USDA policy makers and considered the

evaluation presented in Chapter 2. This chapter is formatted to follow the NSU's *Surveillance and Data Standards for Veterinary Services.*

Chapter 4 examines the effect of exposure of central nervous system tissue to warm temperatures on the immunodetectability of protease-resistant prion protein in elk affected with CWD. This work was initially pursued following the completion of chapter 2, where concerns were presented regarding the accuracy of diagnostic assays for TSE diagnosis when applied to tissue affected with post-mortem or post-collection tissue decomposition, and regarding the diagnostic strategy used for such tissue by TSE surveillance systems. The results of this work are additionally valuable for exploring methods that may be effective at disposing material contaminated with TSE-agents. This paper was formatted for *Veterinary Research.*

Chapter 5 presents an evaluation of prion protein immunohistochemistry applied to recto-anal mucosal lymphoid tissue biopsies for antemortem scrapie diagnosis in sheep and goats. This chapter addresses the potential for this test to improve the diagnostic approach for live animal scrapie diagnosis, and discusses how this test may be used to enhance scrapie surveillance and control programs in the United States. This chapter was written prior to the conclusion of the study; therefore, results presented reflect preliminary findings. However, the conclusion of the study will occur in < 1month following this chapter's production and is expected that changes to data and results will be negligible. This chapter was formatted as a template for a manuscript that will be submitted to the *American Journal of Veterinary Research.*

Chapter 6 provides a summary of the conclusions from Chapters 1 through 5.

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CHAPTER 1

Transmissible spongiform encephalopathy surveillance in the United States

Abstract

Animal disease surveillance systems are needed to protect and improve animal and human health and to promote agriculture-based commerce. Effective systems are founded on comprehensive methods that have been designed to meet pre-defined objectives. These objectives channel results of disease-monitoring into actions or products that are needed by the public and for animal health. Substantial public resources are invested to successfully execute systems that address diseases with serious economic or public health impacts, such as transmissible spongiform encephalopathies (TSEs). The present document describes surveillance systems for bovine spongiform encephalopathy, chronic wasting disease, and scrapie implemented in the United States. These systems have represented the coordinated and collaborative efforts of a range of private and government groups. TSE surveillance has been used to enhance agriculture-based economy by assuring trading partners of the safety of products derived from indigenous livestock. Furthermore, TSE surveillance has been an essential component of integrated disease control programs. Over time, TSE surveillance objectives have evolved as a result of changing socioeconomic pressures; these pressures can result in large investments for disease monitoring approaches that have poorly-defined objectives and consequentially uncertain products. TSE surveillance methods have adjusted as knowledge regarding epidemiology and pathology of disease has advanced. TSE

surveillance has been further improved through the application of surveillance system standards and the process of surveillance system evaluation. As long as TSEs remain a public concern, TSE surveillance systems will be in demand and will benefit from public education and the contributions of veterinarians and livestock producers.

Review of Animal Disease Surveillance Systems

Disease monitoring systems are conducted to assess and measure the disease status of a population.[1] These systems use surveys, a type of cross-sectional study, to describe the frequency and distribution of disease and/or associated risk factors, and in some cases evaluate for geographical or temporal disease trends. Disease surveillance systems combine tools such as monitoring efforts to consequentially prompt and direct some form of action.[2] Surveillance is a continuous process of data collection, analysis, interpretation, reporting, and action. The overall goal of any surveillance system is to increase understanding of the occurrence of disease and to improve animal health through surveillance results-directed actions.

An inclusive surveillance system has clearly defined objectives and plan of action for each potential outcome. A comprehensive surveillance program provides the infrastructure and resources to meet its stated objectives. Completion of testing on a critical quantity of samples does not constitute a surveillance system. For a surveillance system to generate useable and valid results, standardized methods must be formulated. Methods should address sampling strategy, appropriate level of sampling, characteristics

of the target population (the population susceptible to disease for which statistical inference will be made), triggers for sampling an animal of the target population, selection of data sources to enroll in the program, data collection practices, protocols for transmission of data, and case definitions. Case definitions are usually based on the results of laboratory testing, but may require the presence of certain patterns of clinical findings. For example, "syndromic surveillance" monitors health-related data, such as a combination of certain clinical signs that are evident before a diagnostic strategy is implemented and are associated with a degree of probability for disease that warrants further diagnostic investigation. Using this type of case definition facilitates early recognition of certain disease outbreaks. When sampling from the entire population is not practical, sampling methods may use indirect approaches to measuring disease in a target population. For example, "sentinel surveillance" collects data from certain institutions that are believed to serve as a proxy for monitoring disease in the entire target population. These sites may be selected based on availability, reliability, location, or the characteristics of their associated population subgroups. To increase the efficiency with which disease can be detected, "targeted surveillance" focuses sampling efforts on a subpopulation ("targeted population") that has a higher prevalence of disease, or greater risk for disease, than the target population.

Most surveillance programs collect data actively, that is, the target population is sought and evaluated directly to meet the needs of the surveillance program. However, data that are passively generated for primary purposes other than surveillance may be acquired through retrospective or continuous review of institutional databases or through voluntary

or required reporting of certain diseases or obvious outbreaks. Such data are typically generated at the discretion and intuition of producers, veterinary practitioners, and diagnosticians when testing of clinically suspicious animals is pursued. Therefore, the ability of passive surveillance to identify disease is limited by the awareness of the professionals involved, fears associated with consequences of test results, and by available diagnostic support. Inclusion of passively collected data may be most important for surveillance systems intended to detect diseases that are unexpected or uncharacterized, or diseases of low public interest that are allocated limited resources.

Animal disease surveillance programs provide several products intended to meet public need for stable agriculture-based economy and protection of animal and human health. Major products of surveillance can be divided into the following primary categories: contribution to disease control programs, detection of serious diseases, and trade facilitation.

1. Contribution to Disease Control Programs

In order to direct treatment or preventative measures that reduce the occurrence of a disease, knowledge regarding the amount and distribution of a disease in a population is needed. Thus, to reduce the frequency of a specific disease over time, disease control or eradication programs strategically institute interventions based on surveillance results. For example, efforts to mitigate a disease's transmission can be more effectively focused after surveillance has identified infected individuals and cohorts. In some situations, surveillance results can be

used to prevent the exposure of naïve animals or humans. For instance, movement restriction may be placed on a diseased animal or herd, or products derived from diseased animals may be made unavailable to consumers. Furthermore, temporal and comparative analyses of disease trends are sometimes used to determine whether established interventions have been successful. Surveillance programs can also advance the understanding of a disease's epidemiology and ecology. By collecting supplemental data pertaining to a host or hazard, hypotheses regarding factors associated with disease occurrence may be elucidated. These results may provide the basis for novel intervention strategies.

2. Detection of Serious Diseases

Developed countries invest heavily in livestock production management technologies and infrastructures that result in efficient and high yield per animal. For this reason, the agriculture-based economy of these countries is extremely vulnerable to small changes in output per animal. Outbreaks of livestock disease should be considered emergencies, since widespread establishment of even mild disease has the potential to cause profound economic impact.

The threat of emerging and re-emerging infectious animal disease is an internationally increasing concern. Emerging pathogens are opportunists that capitalize on disruption in the balance between host and pathogen ecologies. Current global circumstances that contribute to an increased rate of disease

emergence include escalation in international movement of humans, animals, and animal products, encroachment of uncultivated environment by civilization, climatic change, and intensification of agricultural production systems.[3-5] New pathogens tend to spread rapidly within a naïve population and are frequently zoonotic.[3] Therefore emerging diseases often warrant liberal preventative or control measures that are a substantial burden to agriculture-based economy.

The threat of transboundary infectious diseases is also of increasing concern. The perceived risk for introduction of these diseases has increased as a result of globalization and the use of agroterrorism. Extensive restrictions on imports of animals and animal products are often placed on countries when an emerging or transboundary disease becomes established within its resident animal population. These restrictions may cause considerable economic hardship and are largely driven by consumer and government reactions. Early recognition and response is essential to prevent such emergencies from escalating to a social and/or economic disaster. To detect early changes in an animal population's health status, a reliable animal disease surveillance system is needed.

3. Trade facilitation

Surveillance findings often estimate or establish the level of disease that is present in a certain population, and sometimes document the distribution and spread of disease. This information is useful for conducting an accurate risk assessment that addresses the impact of the present occurrence of disease or the potential

impact of disease if introduced. Moreover, these findings are needed to certify low risk for the occurrence of certain diseases that are associated with international trade sensitivity. The Sanitary and Phytosanitary (SPS) Agreement of the World Trade Organization (WTO), established in 1995 to reduce non-tariff barriers to trade, stipulates that protective measures applied to imports must be founded on scientifically valid concepts and must be motivated by the protection of consumer and animal health.[6] The SPS Agreement recognized the World Organization for Animal Health (Office Internationales des Epizooties [OIE]) as the organization responsible for establishing international animal health standards. The guidelines provided by the OIE International Animal Health Code asserts that a country can only establish freedom from a disease if an adequate surveillance program for that disease exists.[7,8] To satisfy trading partners, the surveillance programs used by a country to assess and substantiate its animal health status must conform to qualitative and methodological surveillance standards set forth by the OIE.[7]

Given the public need for animal disease surveillance, these programs are usually overseen by the agencies that comprise a nation's veterinary statutory body. The prioritization of diseases for which surveillance will be conducted, and the allocation of resources to that system, usually involve an intuitive or purposeful cost-benefit analysis that may consider the probability of the event of interest, and the severity of public health, economic, and trade consequences of the event.[9] The consequences associated with a disease may be influenced by several factors including level of understanding

established for the agent or disease, epidemiological characteristics of the disease (e.g. transmissibility, availability of susceptible hosts and vectors, range of species affected, existence of carriers), effectiveness of existing diagnostic strategies, degree of associated livestock production losses, costs associated with controlling disease, and public perception.

Animal disease surveillance programs in the U.S. embody the combined efforts of federal, regional and state agencies, universities, and various stakeholders of the private sector. Especially pivotal to the oversight of national surveillance activities in the U.S. are the Centers for Epidemiology and Animal Health (CEAH), a part of the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS). The divisions of CEAH most invested in animal disease surveillance are the National Surveillance Unit (NSU) and the National Animal Health Monitoring System (NAHMS). The NSU is devoted to developing, evaluating, and enhancing surveillance programs for animal diseases prioritized by VS, and for integrating, analyzing and reporting results derived from these programs. The NAHMS focuses on surveys that address the health and related management strategies of domestic livestock in the U.S. Implementation of surveillance plans is overseen by and enlists the resources of VS. VS is responsible for providing resources, infrastructure, and oversight necessary to implement a surveillance plan. Since a number of regional or federal jurisdictional agencies may undertake surveillance for an animal disease in the U.S., several disparate animal disease surveillance systems have been established. VS

instituted the National Animal Health Surveillance System to help coordinate the collective surveillance efforts of the nation.

Veterinary Services relies heavily on the support of the National Veterinary Services Laboratories (NVSL) to complete the laboratory testing crucial to most animal disease surveillance programs. In addition to diagnostic service for domestic and trans-boundary diseases, the NVSL is the national reference laboratory, providing veterinary diagnostic laboratories within the U.S. with technical guidance, training, and quality assurance oversight. NVSL contracts laboratories for participation in surveillance testing and conducts confirmatory testing on screen-positive samples for certain diseases. This decentralized approach expedites testing while ensuring that testing is performed for its intended purposes and that oversight is in place to endorse validity of test results. Contracted diagnostic laboratories have proficient animal health specialists, appropriate equipment, comprehensive and functional databases, communication or reporting systems, and adequate capacity to fulfill their role in meeting a surveillance program's objectives. Diagnostic kits used for animal disease control programs are approved and regulated by the Center for Veterinary Biologics (CVB) section of VS. NVSL and contracted laboratories may base the selection of an approved diagnostic assay on several factors: characteristics of the assay's performance; profitability and laboratory throughput; practicality and technical support; degree to which the assay meets standards described by the OIE.[10] The National Animal Health Laboratory Network (NAHLN) was established in 2002 to facilitate coordination between federal, state, and university laboratories which provide testing services for animal disease control programs. The

NAHLN is overseen by NVSL and is a partnership between the USDA's Cooperative State Research, Education and Extension Service, and the American Association of Veterinary Laboratory Diagnosticians. Formation of the NAHLN has nationally improved consistency, accuracy, accessibility, and timeliness of test results for surveillance programs by organizing laboratories into groups best suited to address certain testing needs.

Transmissible spongiform encephalopathy (TSE) surveillance activities conducted by the U.S. demonstrate the range of objectives a nation's surveillance programs are capable of addressing, and the variety of related challenges that may be presented. The history of TSE surveillance activities in the U.S. exemplifies the need for conducting nationally coordinated programs and underscores the cooperative nature necessary for their achievement. When examining the evolution and maturation of TSE surveillance in the U.S., several fundamental concepts of disease surveillance are highlighted including: surveillance goals adjust to changing public needs; methods of surveillance are tailored to a program's goals and resources; and monitoring systems are continuously improved by scientific advancements in understanding or recognizing disease.

Transmissible Spongiform Encephalopathies

The TSEs comprise a group of universally fatal neurodegenerative diseases thought to be caused by an infectious proteinaceous particle, termed prion.[11] The TSEs include Crutzfeldt-Jakob disease (CJD) of humans, bovine spongiform encephalopathy (BSE) of cattle, scrapie of sheep and goats, and chronic wasting disease (CWD) of deer, elk, and

moose. Scrapie was the first recognized mammalian TSE. Identified more than two centuries ago, it presently exists around the world, accept in New Zealand and Australia. BSE and CWD emerged within the last few decades.[12,13] The emergence of BSE coincided with reduced use of organic solvents in the production of meat and bone meal through the rendering process.[14] The origin of BSE agent is unresolved and possibly involves scrapie agent crossing the species barrier or spontaneous genetic mutation of the bovid prion protein gene.[15,16] The factors involved in the emergence of CWD agent remain unknown as well.

BSE is transmitted by feeding meat and bone meal derived from ruminants infected with BSE-agent.[17] In contrast, scrapie and CWD are contagious; these agents are transmitted horizontally, although the specific mechanisms involved are unknown.[18,19] As prions may persist in the environment,[20] it is possible that transmission of scrapie and CWD is indirect. Pathogenesis studies seem to indicate an oral route of infection exists.[21,22] Sources of exposure remain unknown. Saliva, blood, excrement and decomposing carcasses may be sources of CWD agent.[23,24] Feces and placental or fetal tissues may be sources of scrapie agent.[25]

TSEs are characterized by the accumulation of protease-resistant disease-specific isoform of the host-encoded prion protein (PrP^{res}) in certain tissues and are clinically evidenced by weight loss, behavioral abnormalities, and neurologic deficits. Animals are most susceptible to infection in the first few months of life, but clinical disease is not evident until years later; animals usually die as adults, weeks to months following the onset of

clinical signs.[19,26-28] For scrapie and CWD, susceptibility and course of disease may be influenced by certain polymorphisms in the prion protein (PrP)gene.[29,30]

At or around the onset of clinical signs, TSE diagnosis can be accomplished by histological identification of characteristic vacuolar degeneration within central nervous system (CNS) tissue. PrP^{res}, the only presently recognized biomarker for disease, accumulates prior to the development of these lesions and can be detected in subclinically affected animals. However, PrP^{res} accumulation is unfortunately not detectable early in the incubation period and therefore its detection is usually not accomplished in young infected animals. Infection status can be classified much earlier in the disease process by evaluating tissues for infectivity rather than molecular or microscopic features of disease; however, the time and expense associated with bioassay seems to restrict its use to laboratory-based research.

PrP^{res} may be detected by immunohistochemistry, Western blot/scrapie-associated fibril (SAF) immunoblot, immunosorbent assays ("rapid tests"), or by electron microscopic identification of SAFs (which are comprised largely of PrP^{res}). Accumulation of PrP^{res} in CNS tissue is a hallmark of disease; in TSE-affected sheep and cervids, PrP^{res} deposition may be detected first in lymphoid tissue.[22,31,32] The CNS site preferred for testing is the medulla oblongata at the level of the obex since this area contains brainstem nuclei which seem to have consistent early PrP^{res} accumulation.[33-35] However, CNS PrP^{res} accumulation in scrapie-affected animals can be multifocal, and in cases of atypical scrapie, PrP^{res} may initially accumulate in cerebral cortex and cerebellum.[36,37] Therefore,

testing other CNS sites in addition to obex may improve the accuracy of a diagnostic strategy for scrapie. PrP^{res} immunodetection in tonsil and recto-anal mucosa-associated lymphoid tissue (RAMALT) has been used to accomplish antemortem CWD diagnosis.[38,39] PrP^{res} accumulates less consistently in tonsils and lymph nodes of CWD-affected elk than deer.[40] Therefore, little confidence regarding an elk's disease status is gained from negative results when testing these tissues. PrP^{res} immunodetection in third eyelid,[41] tonsil,[42] and RAMALT has been used to accomplish antemortem scrapie diagnosis.[43] However, caution must also be used when interpreting negative results derived from testing lymphoid tissues in sheep since accumulation in these tissues may not occur in certain breeds or in sheep with certain PrP genotypes.[31,44-46]

To determine whether an assay for TSE diagnosis is appropriate for use in the context of a surveillance program, an assay's performance should be considered in terms of diagnostic sensitivity (proportion of diseased animals that test positive) and specificity (proportion of non-diseased animals that test negative). To correctly classify an animal's disease status, such that the accuracy of surveillance results is optimized, highly sensitive assays that have rapid turn-around time are often selected as screening tests, and highly specific assays are often selected as confirmatory tests. The OIE considers assays for PrP^{res} immunodetection to be highly sensitive for detecting animals with clinical disease.[47,48] Western blot and IHC are considered to have near perfect specificity and are preferred confirmatory tests. Given the high-throughput capacity and rapid completion of immunosorbent assays, these are usually selected as screening tests; several have been extensively evaluated for use in the EU and have been found to have sensitivity that

approximates IHC and Western blot.[49-51] Thus, when a rapid test used in a TSE surveillance program produces a positive result, the animal's TSE status is determined by the results derived from testing the sample again with a confirmatory test such as IHC or Western blot. There is debate as to which confirmatory test should be considered the criterion-referenced standard. IHC allows the pathologist to confirm that the correct microanatomic location of the obex is being evaluated, evaluate for the presence of supportive microscopic lesions, and ensure that the morphological features and distribution of PrP^{res} immunostaining is consistent with a TSE. IHC requires a clinically skilled pathologist to interpret results. Conversely, a larger volume of tissue can be evaluated for the presence of PrP^{res} using the Western blot than with IHC; this is a particular advantage since PrP^{res} distribution in the brainstem may not be homogenous. Histology is not considered an acceptable stand-alone screening test since its sensitivity is less than methods which detect PrP^{res} accumulation and since lesions may not be prominent in animals with clinical disease.[52] Therefore, when histology is negative for a TSE, it is appropriate to test for PrP^{res} accumulation before classifying an animal as non-diseased. Furthermore, the specificity of histology is suboptimal since perikaryonic vacuolation of neurons, indistinguishable from those that occur with a TSE, have been recognized in healthy cattle and sheep.[47,48]

Most TSEs have a very limited species range; however, evidence implicates BSE agent as the cause of variant CJD in humans.[53-55] This discovery in combination with the detriment that the BSE epidemic was to the European cattle and rendering industries have caused TSEs to gained considerable public attention. The general public is considerably

apprehensive about the risk that BSE-infected cattle pose to food safety. Furthermore, there is concern for other TSEs to cross the species barrier. For example, zoonosis of CWD agent has been suspected in cases of CJD occurring in young patients who had consumed venison,[56-58] although supportive epidemiologic evidence is presently lacking. Existence of a TSE in an animal population generally results in substantial impacts on international trade that are largely dictated by public perception.

BSE Surveillance System

BSE emerged in Great Britain in 1985 and was subsequently recognized in most European countries, Japan, and Canada.[13] Since the origin of BSE agent was unknown, there was concern that other animal TSEs existing in the U.S. (including scrapie, CWD, and transmissible mink encephalopathy) may have the potential to cause BSE in cattle. Furthermore, there was the potential for U.S. cattle to be exposed to BSE-agent through imported meat and bone meal. To subdue speculation that BSE was present in the U.S. cattle population,[59,60] in 1990 compulsory notification of BSE was instituted and the U.S. began testing clinical suspects for BSE.[61] The testing initiative addressed cattle 2 years of age or older that were affected with neurologic disease or that had tested negative for rabies. In 1993, testing efforts were expanded to include nonambulatory ("downer") cattle. All testing was conducted by NVSL and involved histologic examination of obex. In 1995, testing strategy was changed to use newly developed PrP IHC on obex.[62] From 1991 to 2000, the U.S. tested between 175 and 5,272 cattle per year. In 2001, BSE testing requirements were established for all member states of the European Union

(EU).[63] In 2002, the USDA increased testing efforts to evaluate 20,000 cattle per year, including adult cattle that die on-farm.

In December of 2003, a nonambulatory 6 year old Holstein dairy cow residing in Washington state, that had been imported from Canada in 2001, was sampled at a slaughter facility and tested positive for BSE.[64] The response that ensued exemplified that even diseases with very low morbidity may result in implementation of rigorous control measures and severe trade consequences if there is limited understanding of the agent and/or if the agent has zoonotic potential. Fifty-three countries banned imports of U.S. cattle and beef products; related losses have been estimated to surpass $3.2 billion.[65] The measures that had been instituted by the U.S. to prevent the introduction and spread of BSE were considered to be effective.[66] These included a ban on importation of ruminants and at-risk ruminant-derived products from BSE-endemic countries (9CFR94.18), and the prohibition of feeding certain mammalian-derived proteins to ruminants (21CFR589.2000). However, an international review panel commissioned by the U.S. recommended that BSE testing efforts be significantly extended so that the magnitude of the presence of BSE could be established and the potential need for more aggressive control measures could be recognized.[67] To more accurately determine the level of disease present in the U.S. cattle population, such that trading partners could be assured of the U.S. BSE status and informed internal decisions could be made for BSE control policies, the "enhanced" BSE surveillance system was implemented from June, 2004 through March, 2006. Also in response to the BSE case recognized in 2003, the U.S. established several measures to protect the human food supply including exclusion

of nonambulatory cattle from slaughter, removal of "specified risk materials" (tissues known to contain BSE agent in infected animals)[68] from meat for human consumption (Docket No. 03-025IF), prohibition of the use of injection stunning devices to immobilize cattle during slaughter (Docket No. 01-033IF).

The methods adopted by the enhanced surveillance system were designed to extend testing efforts in order to detect BSE in the adult U.S. cattle population, if it was present. Methods for sampling to detect disease usually approximate sampling goals which consider a specified design prevalence (detection limits; selected level of disease that is believed to exceed true prevalence), the size of the target population, and the desired degree of statistical confidence (the probability that at least 1 disease-affected animal would be found if true prevalence is equal to or greater than the design prevalence).[69] The number of samples that must be tested to detect a disease believed to be extremely rare in a cattle population that approaches 42 million exceeds financial and logistical practicality.

To reduce the number of samples needed to detect disease, and to efficiently allocate surveillance resources, a targeted sampling strategy was used.[70] The targeted population is defined by characteristics (risk factors) that are associated with a higher probability of disease. Since the targeted population is more likely to contain diseased animals than the target population, a lower sample size is needed to detect disease. The targeted population selected by the enhanced system consisted of cattle of any age that were clinically suspicious for BSE, or cattle 30 months of age or older that were

nonambulatory, dead of unknown reason, had other clinical signs that may be associated with BSE, or were condemned at slaughter during antemortem inspection. These characteristics are included in sub-populations of cattle known to have a higher prevalence of disease.[71] The size of the targeted population was estimated to include 445,886 cattle based on reported estimates for on-farm cattle mortalities, adult cattle condemned at slaughter, and the number of foreign animal disease investigations historically addressing cattle with central nervous system signs.[72-74] Considering this population size and assuming that all cases of BSE would occur in this population, a detection limit of 1 case of BSE per 10 million adult cattle was used to determine that if a minimum of 268,500 targeted animals were tested and no positive animals were identified, the U.S. could be 99% confident that BSE was not present.[75]

This sample size estimate assumed that animals of the targeted population would be sampled randomly. The inherent difficulty in identifying every animal of the targeted population, the non-existence of legal requirements to report targeted cattle, and the voluntary nature of animal disease surveillance programs in the U.S. preclude random sampling. Therefore, locations that were believed to have the greatest access to the targeted population were enlisted to identify and sample targeted animals. These locations included rendering facilities, salvage slaughter facilities (those that accept meat from dead, dying, disabled, or diseased animals that is unfit for human consumption), all federally-inspected slaughter facilities, on-farm, veterinary diagnostic or public health laboratories, veterinary practices, and livestock auctions. In attempt to minimize the effect of nonrandom sampling, a census-based strategy was adopted where any animal

fitting the targeted population that presented to any location participating in sample collection was sampled. The vast majority of samples evaluated by the enhanced system were collected by rendering and salvage slaughter facilities.[76]

In response to the substantial testing demands of the new surveillance system, five rapid tests were approved by the CVB. The NVSL contracted several veterinary diagnostic laboratories to conduct screening tests; all laboratories elected to use the same assay (BioRad TeSeE). Confirmatory IHC was conducted by NVSL. This test strategy conformed to OIE standards,[77] and its sensitivity was comparable to the strategy used by the EU.[63] In 2005, the NVSL added Western blotting as an additional confirmatory test to be used in parallel with IHC, after a sample that was positive by rapid test and negative by IHC was confirmed as BSE-positive using Western blot.[78,79]

The enhanced surveillance system tested 647,045 targeted cattle. This level of sampling exceeded what was necessary for the system to meet its stated objectives, and negates concerns of inadequate sampling. Furthermore, the level of BSE surveillance conducted in the U.S. from 1999 to 2006 far surpassed OIE standards.[76] The OIE code supports a targeted strategy by assigning point values to samples evaluated from animals of certain sub-populations based on the likelihood of testing positive for BSE.[80] These sub-populations, or "surveillance streams", include clinical suspects, casualty slaughter, fallen stock and healthy slaughter. Analysis of the data collected by the enhanced system involved assigning samples to these "surveillance streams" according to the criteria that defined an animal as targeted, and the likelihood of recorded clinical signs being

associated with BSE.[76,81] Compiled points generated according to the OIE standards evidenced to trading partners that Type A surveillance (surveillance associated with controlled BSE risk status) had been accomplished in the U.S,[76] and the U.S. subsequently established controlled risk status with the OIE.

The enhanced surveillance system identified 2 positive cattle: a nonambulatory 10 year old cross-bred beef cow sampled by a veterinarian on-farm in Alabama,[82] and a 12 year old Brahma-cross beef cow residing in Texas that died during transport to a slaughter facility and was subsequently sampled at a pet food plant.[78] The molecular features of the Texas case were consistent with atypical BSE cases which accumulate PrP^{res} with unusually high molecular weight of its unglycosylated isoform.[79] Presently, it is unclear if this type of BSE represents a rare sporadic form of disease with questionable transmissibility, or a variant of disease caused by a novel strain of BSE agent.

One drawback of a targeted sampling strategy is that the bias intentionally created to detect disease complicates the methods needed to estimate prevalence, if such estimation is the primary purpose of the surveillance system. Statistical adjustments based on various assumptions are required to infer probabilistic measurements of disease occurrence to the target population. Two statistical models were used to examine surveillance data produced from 1999 to 2006; results indicated that the prevalence of BSE in the U.S. was extremely low (less than 1 infected animal per million adult animals).[83]

Following the conclusion of the enhanced system, BSE surveillance interests in the U.S. were subsequently re-directed towards efficiently assuring that safeguards established to prevent introduction and transmission of BSE remain effective. Furthermore, the new "ongoing" system is intended to maintain assurance of trading partners of the U.S. BSE status. The plan for the ongoing system was documented using newly established surveillance standards and guidelines of the NSU.[84] These guidelines resulted in a plan that reflects predefined objectives, comprehensively details surveillance methods, and facilitates surveillance system evaluation.

Data acquired through the enhanced system was used when adjusting BSE surveillance design to address the new objectives. The majority of OIE surveillance points produced during the enhanced system were derived from cattle classified as "clinical suspects" or "casualty slaughter". Therefore, to improve the sensitivity of surveillance, the ongoing system maintains a targeted sampling but further focuses sampling efforts on cattle belonging to these subpopulations that were most productive for the enhanced system.[85] Efforts for collecting clinical history, data that are needed to capture clinically suspicious cattle, have been augmented; at least one clinical sign must be recorded for each sampled animal. Since the smallest number of clinical suspects was collected by rendering and salvage slaughter facilities during the implementation of the enhanced system, involvement of these collection sites has been drastically downsized. On-farm sample collection has been prioritized since veterinarians and those who handle cattle on a daily basis are best suited to recognize and document subtle clinical signs that are potentially associated with BSE. Similar to the enhanced system, all targeted animals presenting to

surveillance are sampled; however, statistically determined sampling goals needed to maintain confidence that BSE prevalence remains below 1 case per 1 million adult cattle require only 40,000 cattle. The transition to the ongoing surveillance system began in July of 2006; to present, no BSE-positive cattle have been identified.

BSE surveillance systems of some countries include testing apparently healthy animals at slaughter in the interest of removing animals with BSE from the human food chain. This is not done in the U.S. for several reasons. First, a negative test result does not necessarily indicate that food is safe since diseased cattle may not have positive IHC results until late in the incubation stage. The U.S. alternatively focuses resources on the food-safety measures described above; these are highly effective at excluding BSE-infected material from human consumption. In terms of disease monitoring for trade purposes, it is much more productive to invest surveillance resources into testing animals belonging to subpopulations associated with higher prevalence of BSE than healthy cattle at slaughter.

CWD Surveillance Systems

CWD was first recognized in 1967 in captive deer of several wildlife facilities in Colorado and Wyoming.[12] In 1981, CWD became the only TSE known to affect free-living species when it was recognized in free-ranging deer and elk in Colorado;[86] however, it is unclear whether CWD arose first in captive or free-ranging cervids. CWD was subsequently recognized in wild cervid populations of other regions of North America, including Wyoming, Utah, New Mexico, Nebraska, Kansas, South Dakota,

Wisconsin, Illinois, West Virginia, New York, Saskatchewan, and Alberta. In 1996, CWD was identified in farmed elk; these belonged to a herd located in Saskatchewan. It followed that CWD was identified in commercially-raised cervids of several areas of North America including Alberta, South Dakota, Wisconsin, Minnesota, Nebraska, Oklahoma, Colorado, New York, and Montana. Most affected herds have been depopulated; the others remain under quarantine. The apparent national spread of CWD in free-ranging cervids seems to have been associated with movement of farmed cervids. The prevalence of CWD in free-ranging cervids has been estimated to range from 0.5-5% in Colorado and Wyoming.[87] Reported prevalence estimates for CWD have been higher in deer than elk, and substantially higher in captive than free-ranging cervids.[19,87,88]

CWD has been transmitted to cattle through experimental intracerebral inoculation;[89] however, CWD appears to pose no natural risk to cattle. A targeted survey of CWD-exposed cattle failed to detect cattle with spongiform encephalopathy,[90] and PrP^{res} isolated from index indigenous cases of BSE in North American cattle is molecularly dissimilar to PrP^{res} isolated from cervids affected with CWD.[79,91] Nonetheless, the presence of CWD in commercially-raised cervids has caused significant economic losses to U.S. agriculture. The farmed venison and antler velvet markets of the U.S. and Canada have been devastated by trade restrictions affecting export of cervids and their products and by reduced demand for these products due to public perception of the risk for humans to develop disease following exposure to CWD agent. Furthermore, given the apparently rapid national dissemination of disease, and epidemiologic models that suggest unmanaged CWD could lead to extinction of infected deer populations,[87] there is alarm

that the CWD epidemic will be a detriment to tourism and recreational industries that depend on native deer and elk resources. These matters have warranted the development of surveillance-dependent disease control measures aimed at decreasing the occurrence and limiting the spread of disease.

Regulatory authorities for CWD surveillance vary by state. In general, state and federal game and wildlife management agencies have been responsible for monitoring and controlling CWD in wild cervids, whereas state and federal agricultural agencies have been responsible for captive commercial deer and elk. Until recently, surveillance activities for CWD were completed largely at the discretion of state agencies. Prior to 2002, CWD surveillance of free-ranging cervids was largely conducted by states that were endemic with CWD, states where a wild cervid population contributes significantly to recreation-based economy, or states where CJD had been diagnosed in hunters. The recognition of CWD in free-ranging deer of states east of the Mississippi River, and the transport of CWD-exposed commercial elk to states across the country, called attention to the need for a national control program that was capable of coordinating the federal and state wildlife, game, and agricultural agencies. At the request of Congress, and through the cooperative efforts of APHIS and the U.S. Department of Interior, national needs and goals for CWD management in captive and free-ranging cervids were identified and described in 2002.[92,93] As part of this initiative, recommendations for surveillance methods for monitoring CWD in wildlife were devised.[94]

It is difficult for a single CWD surveillance plan to fully consider the differences in rationale for surveillance, social and political driving forces for surveillance, available resources, and size of captive and free-ranging cervid populations that occur between jurisdictional regions. In regards to free-ranging cervids, regional differences in geography, species at-risk, and free-ranging cervid ecology further prohibit the formulation of a single comprehensive national plan for CWD surveillance. To promote uniform surveillance across the nation while meeting the needs of individual states and regions, states determined to be at-risk for CWD were tasked to design surveillance programs for their wildlife that conformed to established recommendations.[94] In 2002 APHIS began to appropriate funds for these state-based programs following their approval through the formation of cooperative agreements. Funding priority is given to states where the presence of CWD has been recognized in the indigenous free-ranging or captive cervid population, followed by states that are adjacent to areas identified to contain CWD. Presently, data derived from these surveillance activities are maintained by the corresponding state. To control CWD in farmed cervids, in 2002 APHIS also established a provision of indemnity for infected herds (9 CFR 55). A surveillance-dependent program to certify captive herds as free from CWD was also devised by APHIS at this time and is presently awaiting implementation of the final rule.

Surveillance of Wild Cervids

The objectives of surveillance in a wild-life population vary according to a population's disease status and level of risk for disease. In non-affected populations, surveillance is

used for early detection of new foci of disease, such that interventions can expediently be made to reduce or prevent spread disease. In populations with newly recognized disease, the primary objective of surveillance is to better define the extent of disease occurrence in terms of prevalence and distribution, such that disease control strategies can be directed. This information can additionally be used to protect the farmed cervid industry by directing measures such as preventing exposure of farmed animals to free-ranging animals, enhancing movement regulations and disease monitoring efforts for farmed animals in areas with disease, and regionally focusing producer education. In populations endemic with disease, surveillance is largely used to recognize when interventions are warranted to maintain prevalence below a certain level. Such interventions include, reduction in population density (to decrease transmission and emigration of affected animals) by wildlife agency personnel or by provision of extended hunting opportunities,[95] habitat modification, prohibition of artificial feeding, culling of apparently sick animals, and establishing intra-state restrictions of the translocation of live animals, carcasses, meat and trophies. Furthermore, surveillance of endemic populations is useful for detecting direction of spread, conducting research aimed at advancing the understanding of CWD epidemiology, and evaluating the effectiveness of various interventions.

Since CWD is a rare disease, effective surveillance strategies are intensive and require testing a large number of samples. Adjustments to sample size estimates that appropriately account for the propensity of CWD to cluster further inflate sample size. To reduce the sample size needed, and to allocate resources more efficiently, targeted

surveillance may be used (as discussed for BSE surveillance). A targeted surveillance program for CWD typically involves focusing sampling efforts on populations of high-risk geographical regions, such as those apposing areas known to harbor free-ranging CWD-infected cervids, regions that are in proximity to farmed cervid facilities containing positive animals,[96] or regions that have received translocated animals or carcasses from CWD-infected areas. The regions selected for surveillance also correspond to the location of the target population. Dispersed sampling throughout the entire region containing the target population is recommended since disease clustering occurs. To ensure selected regions are evenly represented, regions are usually divided into subunits. Sampling goals are proportionately allocated to each subunit based on population size or the presence of certain risk factors.

A targeted surveillance strategy may also focus sampling efforts on animals more likely to have disease, such as mature animals or clinical suspects. Typical wildlife CWD surveillance programs only test animals greater 1.5 years of age as evidenced by dentition. Animals with clinical signs consistent with CWD are targeted for selective slaughter and testing in some states. A much higher proportion of CWD-positive animals can be detected when sampling clinical suspects than when randomly sampling animals.[87] An added benefit of targeted sampling is that selective removal of animals with clinical signs may contribute to CWD management in endemic areas. Sampling of clinical suspects is typically completed by wildlife agency personnel and is dependent the general public to recognize and voluntarily report abnormal animals. Human population density, public awareness, the level of effort required to report sick animals, interests of game

management personnel, dis-interest of hunters in forfeiting tags for harvest of a debilitated animal, and potential reclusive behavior of a sick animal may influence the likelihood of sampling clinically affected animals. These sources of bias may complicate estimation of prevalence. Also, given the long sub-clinical period of CWD, focusing sampling efforts only on clinically affected animals may be a less sensitive approach to detecting new foci of disease; therefore, it is currently not recommended that a surveillance program rely solely on testing clinically affected animals.

At present, guidelines do not exist for the length of time surveillance should be conducted to detect new foci of disease. However, it is evident that new foci of disease could go undetected if surveillance was performed for intervals shorter than the incubation period for disease.

Wildlife populations present several challenges that necessitate methodological complexities that surpass those of livestock surveillance programs. As each affected wild species may have differing geographical localization patterns, risk factors for disease, and population demographics, surveillance methods must address each separately. In addition, separate populations of the same species, which may exist within the same region should be independently addressed by surveillance. It is very difficult to collect a random sample from a wild animal population as all individual animals in a target population cannot be identified for random selection. Sampling of wild animals is often opportunistic, including testing hunter-harvested animals, naturally occurring mortalities,

accidental automotive mortalities, animals submitted to diagnostic laboratories, and animals sacrificed as a result of routine jurisdictional free-ranging herd health checks.

Testing tissues collected from the heads of hunter-harvested cervids has historically been essential to completing large-scale CWD surveillance in free-ranging cervids. Heads of harvested animals are submitted by hunters to designated testing sites that are usually established at select regulatory hunter check stations. Hunters may be motivated to contribute to surveillance by the provision of free test results (although a negative test provides little information about an animal's true disease status, a hunter may elect not to consume meat from a positive animal). Sampling by hunters is not random. Measurements of disease occurrence may be biased by several factors: positioning and availability of testing sites relative to the target population, differences between the populations available for sampling during the hunting season and the remainder of the year, differential susceptibility of diseased animals to harvest, land access issues, difficulty in obtaining accurate hunter kill coordinates, regulations influencing harvest of certain ages or sexes, and hunter selection for certain animal attributes. However, substantial bias was not detected in a survey for CWD using these sampling methods.[97] The largest drawback of surveillance systems using hunter-harvest sampling is that the efficiency in detecting disease is probably substantially lower than those that focus sampling efforts on clinical suspects.

Sampling of natural or automotive-related mortalities is limited by the rapid decomposition of brain tissue during the postmortem interval prior to sampling.

Prevalence has been found to be slightly higher in mule deer killed by vehicle collisions than mule deer sampled by various other means (including hunter-harvest, culling of apparently normal animals by wildlife managers, and captured tonsil biopsied deer) pooled together.[98] However, the difference in likelihood for an opportunistically sampled animal to have CWD relative to an animal that has been randomly sampled is generally unknown. Caution must be used when interpreting prevalence estimates derived from surveillance using opportunistic sampling strategies.

The diagnostic approach used by a surveillance plan for CWD surveillance also varies by jurisdictional region. In response to availability of novel commercially available diagnostics, and as a result of the increased demand for surveillance-related testing, five rapid tests were licensed by the CVB. The use of rapid tests in surveillance activities has allowed greater throughput that is especially needed during hunting seasons. Furthermore, the availability of results has been considerably improved, appeasing the demand of hunters who desire test results prior to investing in meat processing. The NVSL has contracted several veterinary diagnostic laboratories to conduct IHC and rapid testing for CWD diagnosis. In addition to expediting testing and reporting of results, decentralization of testing has also allowed regions to select a laboratory according to established relationships and competitive pricing for testing services. Due to species-dependent differences in the consistency of PrP^{res} distribution,[40] most surveillance plans presently test lymph node from deer and obex from elk. Confirmatory testing is recommended but not required. In addition to testing lymph node or obex, IHC applied to tonsillar biopsy may be used for antemortem diagnosis of animals with preclinical

CWD.[37] In attempt to reduce CWD prevalence in Rocky Mountain National Park, the National Park Service of the Department of Interior (DOI), working with the Colorado Division of Wildlife, is conducting live animal surveillance using tonsillar biopsy IHC to identify and subsequently cull subclinically infected animals.[99,100]

The most recent and comprehensive publicly-available results for wild cervid CWD surveillance in the U.S. indicate that from October 2002 through September 2003, 117,715 mule deer, white-tailed deer, and elk were tested for CWD; of these 592 animals tested positive.[101] In addition, approximately 90,000 wild cervids were tested for CWD in the U.S. during the 2003-2004 hunting season.[102] Since commonly used methods for monitoring CWD are opportunistic and inherently inefficient, surveillance information is made available through considerable investment of public resources. However, it is difficult to determine how this information has been used to benefit the public. As free-ranging cervid CWD surveillance data are maintained by the jurisdictional agency responsible for a system's implementation, it is unclear whether CWD monitoring results have effectively been used to direct actions for controlling disease. Furthermore, it is uncertain if popular mitigations for disease are effective at reducing prevalence. Surveillance systems are needed that procure the epidemiologic tools and methodology necessary for the evaluation of various interventions' efficacy. This information is needed to determine if these disease interventions, and the surveillance that prompts them, are cost-effective public investments.

Surveillance of Commercial Cervids

Following the identification of CWD-infected captive elk in South Dakota, surveillance for CWD in captive cervids began in 1997. To date, surveillance activities for CWD in captive cervids have been conducted at the discretion of a jurisdictional region. The primary goal of CWD surveillance in farmed cervids is to identify positive herds in order to mitigate disease and prevent introduction into unaffected herds and areas. At present, captive cervid surveillance comprises testing needed to support established regulatory efforts, testing of animals that are epidemiologically related to a positive herd recognized through regulatory efforts, and monitoring needed to complete regional herd certification programs. When positive herds are identified, established regulatory measures require voluntary indemnification or mandatory quarantine in combination with institution of a herd management plan. The management plan is intended to monitor and reduce the level of disease in the herd and may include testing all natural deaths and slaughtered animals from the herd, genotyping to identify animals at higher risk for disease,[30] weighing animals to detect animals with very early clinical disease, premises decontamination plans, and using live-animal tonsillar or RAMALT biopsy IHC to recognize animals with preclinical disease. Due to political pressure, most herds elect to depopulate with indemnity. In addition, captive herds may be indemnified if they were established within an area where CWD is endemic in wildlife.

Herd certification programs conducted by jurisdictional regions largely correspond to the directives outlined by the national herd certification program proposed by APHIS (Docket No. 00–108–3). Interstate movement of captive cervids, including those

captured from free-ranging populations, is presently regulated by the States, but is typically only allowed for animals from herds that have reached sufficient status by participating in a herd certification program. The proposed federal program will limit interstate commercial cervid movement largely to animals enrolled in the herd certification program as well (9 CFR 81). In general, certified status is believed to enhance value and marketability of a herd's animals and products. The national plan will require participating producers to conform to standards for herd management, including record-keeping and animal identification practices. Enrolled herds must be regularly inventoried, confined within an acceptable perimeter fence, and must report all animals over 12 months of age that die for any reason (including animals of hunting ranches killed on-premises or animals that are sent to slaughter) so that CWD testing can be pursued. Herds are certified as being low risk for CWD after 5 years in the program with no evidence of CWD. The 5-year period was elected based on the probable maximum incubation time for CWD and the belief that an exposed cervid will develop signs of disease or die in less than 5 years. Enrolled herds that are found to contain cervids positive for or exposed to CWD lose their status and can only re-enroll after completing a herd management plan. There is concern regarding the ability of certain captive cervid industries, such as hunting ranches, to meet identification, inventory, and testing requirements of the program given management strategies that preclude capture of live animals and recognition of dead animals.

Testing for all captive cervids through regulatory efforts entails application of IHC to retropharyngeal lymph node, tonsil, and obex in parallel. In the event of a positive result,

IHC results are confirmed by the NVSL. As the use of IHC applied to tonsillar biopsy has been limited by the need for general anesthesia, a practical live animal test is in demand. Studies evaluating the performance of IHC applied to RAMALT for antemortem CWD diagnosis are underway. Antemortem CWD diagnostics are anticipated to improve the sensitivity of disease monitoring methods and to reduce the need for indemnification.

In the last few years, CWD surveillance and integrated control programs of commercial cervids have evaluated approximately 15,000 animals per year.[103] To date, these efforts have identified disease in 41 herds of nine states. Most of these herds have been depopulated, however, four infected elk herds and one infected white-tailed deer herd remain under quarantine. Nevertheless, the potential long-term success of herd certification program-based surveillance seems limited by several factors including, the long preclinical incubation period, the lack of tests that can identify positive animals early in the incubation period, producer disincentive to identify disease within a herd, and incomplete understanding of disease epidemiology and ecology. When positive animals of formerly non-diseased herds are detected by surveillance, the inability to identify potential sources of exposure, or recognize factors that promote infection in exposed animals, may prove to preclude productive epidemiologic investigations.

Scrapie Surveillance Systems

Scrapie was first recognized in the U.S. in a Michigan flock in 1947. Prior to the emergence of BSE, scrapie received little national attention since its occurrence is rare and since animals are affected later in life, after years of productivity. However, since there was little understanding of the cause, origin, transmission, and pathogenesis of scrapie, the risk that this disease posed to the U.S. sheep industry was unknown. As a result, the National Scrapie Control and Eradication Program was started by the USDA in 1952.[104] This program involved quarantine and complete indemnification of flocks from which positive animals were identified. The recognition of scrapie-positive flocks was accomplished using ad-hoc testing of clinically suspicious animals in combination with the establishment of state-based compulsory notification of scrapie-positive animals. Diagnosis was accomplished by histological or immunohistochemical evaluation of brain tissue completed by the NVSL. This original scrapie eradication program also conducted limited epidemiologic investigation and testing to identify the source flock where animals of newly infected flocks may have been originally exposed to scrapie agent.

Following the emergence of BSE, public fears of food-borne zoonosis or of emergence of new diseases originating from existing TSE agents,[16,53] gave rise to the potential for substantial export restrictions of live sheep and goats or small ruminant products from scrapie-endemic countries. In addition, there is potential for unrecognized BSE to exist as an undetected public health threat in a small ruminant population that is endemic with scrapie. This concern has been recently elevated as disease caused by BSE-agent that is

difficult to clinically or pathologically distinguish from scrapie has naturally occurred in a goat,[105] and has been experimentally demonstrated in sheep.[106]

To bolster scrapie control efforts, the voluntary scrapie flock certification program (SFCP) was initiated in 1992, but became firmly established in 1999 (9 CFR 54).[107] The goal of this program was to identify flocks that were free of scrapie. The SFCP allowed producers to improve the marketability of their animals through achieving scrapie-free certification. Similar to the CWD certification program proposed for commercial cervids, certification is obtained after meeting requirements of the program for 5 years without producing laboratory or clinical evidence of scrapie in any animal of the flock. The program requires producers to report sheep or goats with clinical signs suggestive of scrapie, or animals that die on-farm for any reason, to VS such that testing may be pursued. Furthermore, producers must conform to guidelines for animal identification and records management practices. All acquisitions of animals from nonparticipating flocks, or enrolled flocks of lower status, must be reported to VS; addition of such animals may decrease the status of the receiving flock. VS personnel inspect enrolled flocks annually.

Despite existing disease control efforts, in 2001, a questionnaire-based survey administered by the NAHMS to sheep producers in major sheep producing states, revealed that 1.2 percent of operations had suspected or confirmed scrapie during a 3 year period.[108] Furthermore, although animals enrolled in or certified by the SFCP are believed to be economically valuable, the 2001 NAHMS sheep study indicated that

producer awareness of the program was low and that participation was lacking. In 2002, the NAHMS conducted a more focused study that estimated the national prevalence of scrapie in the slaughtered mature sheep population to be 0.20% (SE 0.04).[109]

As scientific knowledge of scrapie advanced, epidemiologic information and new diagnostic tools became available to enhance detection of diseased animals or animals at-risk for disease. The most pivotal diagnostic tools included PrP genotyping and PrP^{res} IHC applied to third eyelid biopsies for antemortem preclinical scrapie diagnosis. Characteristics that designate an animal as having higher-risk for scrapie were better defined, including females of susceptible genotype that are 14 months of age or older, and offspring or members of the contemporary lambing group of scrapie-positive ewes.[110] Genetic susceptibility was characterized by the following polymorphisms of the host prion protein gene: homozygous for alanine or heterozygous for alanine and valine at codon 136; homozygous for glutamine or heterozygous for glutamine and arginine codon 171. By using this knowledge, scrapie control efforts could more efficiently focus testing efforts on high-risk animals, instead of conducting whole-flock indemnification. Furthermore, the live animal test allowed scrapie control efforts identify positive herds without indemnification of all high-risk animals.

These advancements, and the cooperation of the U.S. sheep industry, allowed the establishment of the "accelerated" National Scrapie Eradication Program (NSEP) by VS in 2001.[110] The goal of the NSEP is to eradicate scrapie from the U.S. sheep and goat population by 2011. This program provides guidelines for records management practices

and set forth standards for mandatory animal identification (9 CFR 79). Furthermore, to enforce animal identification requirements, interstate movement restrictions were established for non-identified animals. These measures were established to facilitate epidemiologic investigations. In addition to recognizing source flocks, the NSEP conducts investigations that associate movement of animals from an infected flock with potentially exposed flocks. Most importantly, the NSEP outlines methods to identify scrapie-infected animals in exposed flocks, reduce prevalence in infected flocks, and prevent transmission between flocks. These interventions include indemnity of high-risk animals, genotyping to detect genetically susceptible sheep, and establishment of flock monitoring plans. Flock monitoring plans may include mandatory quarantine, testing of animals that die or are culled, and live animal testing of high-risk sheep. The NSEP has used PrP^{res} IHC applied to a third eyelid biopsy during epidemiologic investigations, as a part of flock monitoring plans when sheep are not indemnified, and to flocks agreeing to test all animals in exchange for flock genotyping. However, since the sensitivity of third eyelid IHC is believed to be less than IHC applied to brain and other lymphoid tissues, animals with negative third eyelid tests must eventually be tested using another tissue to determine scrapie status. IHC applied to RAMALT biopsies is presently under evaluation in the interest of finding a more sensitive and practical antemortem test for scrapie diagnosis.

In contrast to the previous scrapie control program, the NSEP is highly dependent on surveillance activities to identify positive animals and exposed flocks. The goal of scrapie surveillance is to detect as many infected animals as possible so that

epidemiologic investigations and control measures mandated by the NSEP can be directed. Surveillance activities include regulatory scrapie slaughter surveillance (RSSS), the SFCP, and testing of clinical suspects and rabies-negative animals at the discretion veterinarians and veterinary diagnostic laboratories. Furthermore, results of testing conducted during epidemiologic investigations of exposed flocks and results from live-animal testing conducted as part of a flock monitoring plan or in exchange for VS-funded flock genotyping, are typically included in VS reports which describe scrapie surveillance results. Surveillance components are overseen by VS while CEAH provides support for data analysis and report compilation.

Since slaughter is the most accessible avenue to the sheep and goat population, substantial NSEP resources are allocated to RSSS. RSSS represents the majority of scrapie surveillance activities. Similar to BSE and CWD surveillance, RSSS also involves a targeted sampling strategy. Targeted animals include sheep and goats that are dead or down when arriving to slaughter, are clinically suspicious for scrapie, or have a dark or mottled face and are 18 months of age or older. Targeted criteria correspond to subpopulations believed to have a higher prevalence for disease relative to slaughtered sheep as a whole.[109] The annual sample goal for RSSS is 48,000 animals. This goal was based on the number of animals historically sent to slaughter, and the proportion of slaughtered sheep that are dark-faced.[109] However, sampling is census; any animal presented to slaughter that meets targeted criteria is sampled as opportunity allows. All federally inspected slaughter facilities receiving adult sheep participate in the program.

Some states have also contracted state-inspected and custom-exempt slaughter facilities to supplement surveillance efforts.

For RSSS, obex and retropharyngeal lymph node are submitted to contracted laboratories and are tested in parallel for PrP^{res} using IHC. Positive samples are sent to NVSL for confirmatory evaluation of IHC results. All samples from non-white faced clinical suspect sheep are sent directly to NVSL for testing. When samples are positive, remaining obex or lymph node are evaluated for PrP genotype. Animals that are sampled as a result of epidemiologic investigations, or at the discretion of a veterinarian who has determined clinical signs are suspicious for scrapie, are tested by the NVSL for PrP^{res} by applying IHC to obex, tonsil and retropharyngeal lymph node in parallel.

During fiscal year 2006, 116 flocks containing scrapie-positive animals, and 343 scrapie-positive animals were identified through scrapie surveillance and eradication efforts.[111] Approximately 37,000 animals were evaluated through RSSS, resulting in the discovery of 55 positive animals and 27 flocks newly recognized to harbor scrapie-positive animals. Scrapie surveillance systems have considerably empowered the NSEP by recognizing flocks that call for testing, indemnity, and epidemiological investigations. However, it presently is unclear if surveillance focusing on slaughtered sheep and goats will sufficiently support the NSEP such that it is possible to achieve eradication by 2011. The population presenting to slaughter is biased towards older animals whereas most animals with clinical disease are 2-3 years of age.[28] Furthermore, the proportion of flocks that do not send cull sheep to federally-inspected slaughter is uncharacterized. This may be

especially concerning in regards to small flocks that are not monitored by other means, or flocks that sell to ethnic/religious markets which require unique slaughter practices which prohibit sampling of obex.

The NSEP intends to meet OIE requirements needed to designate a country as scrapie-free by 2017.[48] Presently, scrapie surveillance efforts follow general surveillance guidelines recommended by the OIE;[8] however, there is need for a comprehensive written surveillance plan to demonstrate this. The NSU has recently been commissioned to construct a formal plan for national scrapie surveillance efforts that supports the needs of the NSEP. The design will likely follow the NSU's surveillance standards[84] and will consider feedback provided by an evaluation of scrapie surveillance activities that was conducted by the NSU in 2006.[112]

Conclusion

Animal disease surveillance systems are an important responsibility of the veterinary profession, providing the mechanisms to improve the health status of a country's livestock populations and to protect animal and public health. Effective animal disease surveillance systems are needed to safeguard agriculture-based economy and to promote interstate and international commerce. Most animal disease surveillance systems benefit from the veterinarian's ability to recognize clinical signs that are compatible with a monitored disease of interest. Further, surveillance benefits from the role of veterinarians to educate producers and consumers about a disease and how surveillance and other

regulatory efforts will benefit the nation. Veterinarians have an obligation to know the goals of and rationale for surveillance on a local and national level, and should be aware of their potential role in sampling animals, testing clinical suspects, and communicating with agencies responsible for program oversight. As experts in animal disease, veterinarians should actively influence policy-making regarding disease surveillance such that practical and science-based methods are pursued. Veterinarians actively engaged in a surveillance system should be mindful that cooperation and communication facilitate bridging of jurisdictional gaps that are prone to occur with large programs of national significance.

A functional surveillance system is constantly evolving. Surveillance goals should shift over time to address new knowledge of a disease's occurrence and changing socioeconomic pressures. Surveillance methods should periodically be adjusted to account for new information concerning the epidemiology of a disease, especially when a targeted sampling approach is used. Systematic review of a surveillance system's structure and performance should be periodically conducted to provide feedback concerning the quality, efficiency, and success of the system. This feedback, in addition to risk analysis directed towards populations of concern, facilitates a system's improvement and adaptation over time and may help determine whether the public's resources have been sensibly invested.

Research sustaining the development of diagnostic tests should consider the needs of surveillance programs. Surveillance programs are quick to adopt assays that are accurate,

rapid, allow high-throughput, and can be performed on easily collected tissue specimens. Since a variety of factors that affect a test's performance are introduced outside the laboratory, it is important that test evaluation studies consider field conditions presented in the context of a surveillance system. In addition, test evaluations should also take account a test's performance on newly recognized strains or variants of disease.

Veterinary diagnosticians must recognize that data derived from testing on an ad-hoc basis represent a very inefficient approach to identifying a disease of interest, and do not constitute surveillance as results have little value or potential application. Without knowing demographic, geographic, or clinical characteristics of the tested population, the outputs of surveillance are very difficult to interpret. Furthermore, without having an accurate value for the number of animals of the population at-risk that were tested, the denominator necessary to estimate the magnitude of disease remains unknown. Although results may indicate a disease is present, they are unlikely to identify where disease is absent. If any meaningful results are produced from this disease monitoring approach, their validity will be difficult to defend since study design measures that acknowledge or account for potential bias were not in place. Veterinary diagnosticians should work closely with authorities responsible for a program's oversight to ensure that the animals that are tested, the testing strategy used, and supplemental information that is collected, correspond to the needs of a surveillance system.

Resources are logically invested in surveillance systems only when the potential products of the system have been pre-defined. There is little value in determining the distribution

and extent of a disease unless this knowledge will direct certain anticipated actions. Moreover, it is not sensible to spend substantial public resources to monitor for a disease unless findings can and will be used to improve animal and human health or agriculture-based economy. Initiatives to build national, regional, or local surveillance systems must therefore clearly describe the systems' objectives prior to commencing monitoring efforts. Furthermore, the methodological and structural elements necessary for a surveillance system to meet its objectives must be made available such that the public's investment in surveillance is justified. If the resources allocated to a surveillance system are insufficient to provide these necessary elements, one should question whether any disease monitoring should be conducted at all, as intended outcomes will not be produced. When resources are allocated to a continuing surveillance system, evaluations should be conducted to determine whether or not the actions intended by surveillance have occurred, and whether or not these actions have resulted in a product that is useful to the public.

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CHAPTER 2

Evaluation of the United States Enhanced Bovine Spongiform Encephalopathy Surveillance Program

Abstract

A draft protocol for evaluation of animal health surveillance systems was used to assess the U.S. Enhanced BSE Surveillance Program. The purposes of the present evaluation were to test the protocol, to develop a reporting format for such evaluations, and to identify areas of improvement that could be addressed while designing a maintenance plan for BSE surveillance. The evaluation was conducted by a veterinary pathologist and PhD student in veterinary epidemiology. The scope of the evaluation was restricted to assessment of the program's structure, process, and certain qualitative attributes. Most field observations used for the evaluation were gathered during the initial stages of surveillance. The U.S. Enhanced BSE Surveillance Program has been effective in that it has essentially met its stated objectives and has maintained optimal degrees of simplicity, flexibility, timeliness, and usefulness. Efforts should be made by future surveillance plans to improve the acceptability and representativeness of BSE surveillance, with emphasis on improving validity of collected sample data, minimizing geographical bias, and enhancing the capture of animals clinically suspicious for BSE. Additionally, future surveillance plans should adopt a comprehensive format as detailed by the United State's Department of Agriculture – Animal and Plant Health Inspection Service – Veterinary Service's (USDA/APHIS/VS) surveillance standards.

Introduction

Surveillance system evaluation is the systematic review of information pertaining to a surveillance system, with the purposes to determine the extent to which the system meets its stated objectives and USDA/APHIS/VS surveillance standards,[1] and to provide feedback for improving the quality and efficiency of the system. National animal health surveillance systems are prone to inefficiency since they are dictated by complex protocols, are dependent on the cooperation of multiple parties, and since there is a large degree of structural diversity between individual surveillance programs. Periodic surveillance system evaluation facilitates a system's development, improvement and adaptation, and is imperative to assure that public resources are sensibly invested.[2]

The present report has been constructed to summarize findings of the evaluation of the U.S. Enhanced Bovine Spongiform Encephalopathy (BSE) Surveillance Program. The objectives of this evaluation were to test an original surveillance evaluation protocol recently constructed by the National Surveillance Unit (NSU),[3] to develop a reporting format for the evaluation, as well as to review BSE surveillance practices such that improvements could be made while designing a maintenance BSE surveillance plan. Key audiences for this evaluation include NSU personnel, and USDA/APHIS policy makers.

BSE Background

Bovine spongiform encephalopathy, commonly known as "Mad Cow Disease", is a transmissible neurodegenerative disease of adult cattle that emerged in Great Britain in

1985,[4] and has subsequently been identified in cattle of most European countries, Canada, the U.S., and Japan. BSE belongs to the group of transmissible spongiform encephalopathies (TSEs), together with scrapie of sheep, chronic wasting disease (CWD) of free-ranging and captive deer and elk, and Creutzfeldt-Jakob disease (CJD) of humans. TSEs have long latency periods, are untreatable, and currently cannot be prevented by vaccination since there is absence of a host immune response to infection.

Prions are considered the causative agent of TSEs.[5] The nature of the prion remains undetermined. A unique characteristic of the prion is its resistance to inactivation by most conventional physical or chemical decontamination methods.[6] The prion consists mostly of protein, largely comprised by a proteinase-resistant, disease-associated isoform (PrP^{res}) of host-encoded prion protein (PrP^{C}).[5, 7] The pathogenesis of the TSEs requires the formation of PrP^{res} from PrP^{C}.[8] PrP^{C} is a highly conserved cell-surface glycoprotein with unknown function that is abundantly expressed by neural cells.[9] Interaction with PrP^{res} leads to post-translational conformational modification of PrP^{C},[10-12] resulting in its conversion to PrP^{res}.[13, 14] The subsequent pathological accumulation of PrP^{res} in certain tissues defines the TSEs.[15, 16]

BSE is invariably fatal and is characterized by progressive weight loss and neurologic dysfunction. Clinical signs have an insidious onset and typically consist of behavioral changes, apprehension, hyperesthesia, ataxia, and emaciation.[17] Although nonspecific, clinical signs that may be highly suspicious for BSE include hypersensitivity to touch or sound, head shyness, panic-stricken response, kicking in the milking parlor, reluctance to

enter the milking parlor, abnormal ear movement or carriage, increased alertness behavior, reduced milk yield, bruxism, and change in temperament.[18] The duration of clinical signs averages 1 to 2 months prior to death or slaughter, but may range from weeks to a year.[17]

The origin of the BSE agent is unresolved. Theories have considered derivation from a TSE agent of another mammalian species, such as scrapie,[19] or spontaneous genetic mutation of the bovid prion protein (PrP) gene.[20] The emergence of BSE coincided with reduced use of hydrocarbon solvents in the production of meat-and-bone meal (MBM) through carcass rendering.[19]

Transmission of BSE primarily occurs through ingestion of feedstuff, especially ruminant-derived MBM, contaminated with the BSE agent.[17, 21] Dairy breeds are at higher risk for BSE compared to beef breeds since high concentrate feed rations are more frequently used by dairy operations. Most cattle become infected within the first 6 months of life.[22] The mean incubation period for BSE is around 60 months, with clinical onset of disease occurring on average at 4-5 years of age.[23] The age range of affected animals is very wide, although BSE is rarely confirmed in animals less than 30 months of age.[24, 25] Calves born to infected cows have increased risk to develop BSE,[26] especially if born around the time of disease onset in the dam.[27] The risk is most likely influenced by maternal-associated transmission, and to a lesser degree, conserved genetic predisposition. Regardless, a BSE epidemic could probably not be sustained by vertical

transmission alone. Horizontal transmission of BSE between cattle is not believed to occur.

The pathogenesis of BSE appears to involve a much more restricted tissue distribution than other animal TSEs, having reduced involvement of the lymphoreticular system. Following oral challenge with BSE agent, cattle accumulate infectivity in CNS tissue, dorsal root ganglia, trigeminal ganglia, distal ileum, tonsil and bone marrow.[28, 29] These tissues, in addition to spleen, thymus, eyes, skull, vertebral column, and mesentery, are designated as specified risk material (SRM) in cattle > 30 months of age, and are considered to represent the greatest risk of BSE exposure to humans and animals.

At present, no test can be conducted on live animals to detect BSE. Diagnosis is achieved postmortem through examination of central nervous system (CNS) tissue, and is contingent on identification of characteristic histopathologic lesions, detection of PrP^{res}, or electron microscopic visualization of scrapie-associated fibrils.[30] Since PrP^{res} is the only currently known disease-specific macromolecule, all commercially available diagnostic assays rely on its immunological detection.[31] These assays have limited diagnostic sensitivity in that PrP^{res} accumulation is an exponential process, and may not be detectable until late during the incubation period, within months prior to onset of clinical disease.[32] Infected animals that are early in the incubation period can only be identified through demonstration of tissue infectivity using bioassay. The only lesions associated with BSE are found microscopically within CNS tissue.[4, 33] Lesions develop late in the disease process, roughly coinciding with the onset of clinical signs. These

consist of non-inflammatory vacuolar degeneration, or spongiform change, of grey matter and neuronal cell bodies. Astrocytosis and cerebral amyloidosis, features of other TSEs, are unusual with BSE.

For TSEs other than BSE, disease susceptibility and course may be influenced by breed,[34, 35] polymorphisms in the host PrP gene,[36-38] or prion strain. Breed-dependent differential susceptibility or incubation period has not been observed with BSE,[39] and there is little variability in the bovine PrP gene.[40] The consistent neuroanatomical lesion profile in the brains of cattle affected with BSE,[33, 41] and uniform glycoform ratios of PrP^{res} as determined by Western blotting,[42, 43] suggest the existence of a single strain of the BSE agent. However, a recently described atypical form of BSE, termed bovine amyloidotic spongiform encephalopathy (BASE), has modified glycoform patterns similar to sporadic CJD in humans, and may represent an alternative strain of BSE agent.[44]

Although TSEs are usually confined to an individual species, concern has arisen for the potential of inter-species transmission of BSE. The BSE agent has been implicated as the cause of variant CJD in humans, based on epidemiological and mouse inoculation studies,[45, 46] and biochemical PrP^{res} characteristics.[42] In addition, natural exposure to BSE agent has led to similar encephalopathic disease in captive wild ungulates and cats,[47] and in domestic cats.[48]

Regulatory Actions for BSE in the U.S.

The perceived threat to public health and the poorly understood pathogenesis of disease have necessitated widespread establishment of regulatory measures to prevent spread of disease, monitor disease (active surveillance programs), and protect public health. The financial consequences have been severe, especially for BSE-endemic countries. The economic impact of the European Union BSE epidemic has been estimated at €2.75 billion annually, including costs of containment and eradication efforts, safe disposal of animal waste material, preventative measures, animal testing, and losses from reduced value of beef products.[49] Given the economic ramifications, global sensitivity to BSE has prompted significant and rapid effects on international trade. Following the identification of a single BSE-affected imported dairy cow, total U.S. beef industry losses resulting from reduced beef and offal exports have been estimated to range from $3.2 billion to $4.7 billion.[50]

Measures established by the U.S. to prevent new cases of BSE include: ban on importation of ruminants and at-risk ruminant-derived products from BSE-endemic countries, prohibition of feeding of mammalian-derived protein to ruminants, ban on importation of rendered products derived from any species from BSE-endemic countries, requisite that animal feed facilities using ruminant-derived protein become dedicated to production of non-ruminant animal feed, and ban on the feeding of poultry litter to ruminants which may be contaminated with ruminant-derived MBM in the poultry ration. These measures have been considered to effectively reduce the likelihood of BSE introduction and amplification in U.S. cattle.[51-53] Food-safety precautions implemented

by the U.S. to protect the consumer include: exclusion of "down" or dead cattle from slaughter for human consumption, removal of SRM from meat for human consumption, and prohibition of the use of injection stunning devices to immobilize cattle during slaughter.

Reassurance of BSE status to potential consumers or trading partners can be accomplished through achieving "controlled" BSE risk status with the World Organization for Animal Health (Office Internationales des Epizooties [OIE]).[54] To ensure that U.S. cattle commodities pose a controlled risk of transmitting BSE, the U.S. must meet the following OIE requirements: 1) conduction of a risk assessment, 2) implementation of "type A" surveillance practices, 3) accomplish appropriate containment efforts following identification of BSE-affected animals. "Type A" surveillance requires a detection limit of one case per 100,000 adult animals, and a confidence level of 95%. The "type A" surveillance approach uses the relevant adult cattle population size to estimate a desired surveillance point target.[55] The OIE code assigns point values to each sample tested. These values correspond to the likelihood of detecting disease based on sample characteristics, and thus are based on age of the animal sampled, and the subpopulation from which the sample was collected. "Type A" surveillance has been implemented when totaled points exceed the point target, given that surveillance strategy has been acceptable.

Active surveillance for BSE and mandatory notification of BSE diagnosis was initiated in 1990 since the prevalence of BSE in the U.S. was largely unknown. An International

Review Panel (IRP) was commissioned by the USDA, in response to identification of a BSE-affected cow through surveillance efforts in December, 2003,[56] to provide suggestions for control measures that would be justified by the status of BSE in North America. Following recommendations of the IRP, the U.S. Enhanced BSE Surveillance Program was implemented to verify a low prevalence of BSE.[57, 58] The objective of the enhanced surveillance system is to determine whether BSE is present within the native U.S. cattle population, at or above a design prevalence of 1 detectable case per 10,000,000 adult cattle, with 99% confidence. Surveillance data are intended to identify clinically and pre-clinically affected cattle, and trace infected animals to their herd of origin. In addition, surveillance data allow the potential to estimate true BSE prevalence, and demonstrate that U.S. surveillance practices are in accordance with Type A surveillance standards required by the OIE.[55] Furthermore, surveillance data may perhaps assist in the determination of the effectiveness of risk management policies, and, given a rare prevalence for BSE at the chosen design prevalence, the design of a maintenance surveillance plan will be contingent on findings of the enhanced surveillance program.

Methods for Evaluation

The present evaluation of the U.S. Enhanced BSE Surveillance Program has been completed using guidelines provided by the draft, *Protocol for NSU Evaluation of Animal Health Surveillance Systems.*[3] The evaluation process focused on evaluation of surveillance system structure, process and qualitative attributes. Some aspects of the NSU protocol were considered beyond the scope of this evaluation and are not represented in the present document. For example, cost effectiveness is not assessed and the quantitative attributes, sensitivity, and predictive value positive, are not estimated. Additionally, the portion of the Enhanced BSE Surveillance Program which addresses a random sample of clinically normal cattle presented to slaughter is not addressed.

The evaluation was completed by a PhD student in veterinary epidemiology at Colorado State University, and diplomate of the American College of Veterinary Pathologists. This person is considered a non-biased intermediary, who was not involved in design of the surveillance system and who has no personal investment in surveillance findings.

Resources and information pertinent to the program were gathered and reviewed. Several testing facilities and sample collection sites were inspected. A wide variety of persons involved with the system were consulted including those participating in sample collection, sample testing, data entry, data analysis and reporting, and policy making. Field personnel were interviewed to provide descriptions of surveillance processes, as practiced, and opinions on the efficiency and acceptability of the surveillance program,

and to describe problems areas which have been encountered through involvement with the surveillance program. Reported descriptions of the system were compared against direct observations. Characteristics of the surveillance program were contrasted with key components of a surveillance system as described by the draft, *Surveillance and Data Standards for USDA/APHIS Veterinary Services.*[1] The evaluator was advised by APHIS/VS personnel of problem areas which arose during data analysis.

Field observations were completed from July, 2004 to February, 2005. The National Enhanced BSE Surveillance Program adapted several adjustments throughout its term, thus it is possible that some problems presented herein have been resolved, or that new problems have emerged and remain unidentified.

Evaluation of Surveillance Structure, Processes, and Overall operation

Targeted Population

Details: In the interest of efficiency, a targeted surveillance methodology was adopted, whereby only animals at relatively high-risk for BSE would be sampled. Only cattle over 30 months of age, as evidenced by the eruption of at least one of the second set of permanent incisors, were included in the targeted population. The targeted population was classified into several groups based on clinical presentation and collection site:

- Non-ambulatory cattle – includes recumbent ("downer") animals, those that cannot walk, and those that are severely weakened though they may be able to stand and walk for brief periods of times; condition may result from broken appendages, severed tendons or ligaments, nerve paralysis, fractured vertebral column, or metabolic conditions
- CNS signs and/or rabies negative (these animals may be of ANY age) – samples submitted to diagnostic laboratories due to evidence of CNS clinical signs, rabies-negative cases received by public health laboratories, antemortem condemns at slaughter for CNS clinical signs, on-farm "Foreign Animal Disease" investigations for CNS clinical signs or cattle highly suspicious

for BSE

- Cattle exhibiting other signs that may be associated with BSE – cattle that were condemned or euthanized or that died as a result of a moribund condition, tetanus, emaciation, injuries, or non-ambulatory conditions
- Dead cattle – Any dead cattle where the specimen is of diagnostic quality and the cause of death and/or clinical signs prior to death, if known, do not preclude it from the targeted population
- FSIS antemortem condemns (these animals may be of ANY age, but must be >400 pounds at slaughter) – all cattle condemned by FSIS upon antemortem inspection for any reason will be sampled

Strengths:

1. The targeted population addressed by the Enhanced Surveillance Plan is very thorough. All animal characteristics and surveillance streams that may potentially be associated with increased risk for BSE are included in this scheme.

2. The exclusion of healthy adult animals from the targeted population is a sensible allocation of resources, since BSE is unlikely to be identified in these animals and such samples are allotted negligible value by the OIE Code.[55] Detectable cases of BSE are 29.4 times more common in targeted high-risk cattle than in apparently healthy cattle.[59] This approach could provide some benefit to the

surveillance program as it would provide an opportunity to detect disease in targeted animals that evade surveillance through slaughter. However, a very large number of samples would need to be tested to detect disease in these low-risk animals, resulting in a very high cost per case detected. Furthermore, testing healthy animals at slaughter is not a cost-effective measure to improve food safety, because tissues from animals that test negative can still have potential for infectivity. Such an approach would be much less effective than removing SRMs from the food supply. Furthermore, it is unclear if positive test results are associated with infectivity of beef meat.

Areas for Consideration:

3. The targeted population as described by the Enhanced Surveillance Plan is lengthy and ambiguous. Simplicity and clarity in describing the targeted population is necessary to ensure data collection is appropriately initiated and samples are accurately collected from targeted animals. Since data from animals of relatively higher risk have the potential to be analyzed differently, the targeted population should be subdivided into groups according to surveillance streams and associated risk for disease. Specific characteristics that assign an animal to the targeted population (for example, "neurological signs") should not stratify more than one group. If surveillance results will be applied to international models, such as those that

determine adequacy of surveillance results, these groups should be comparable to those used by surveillance programs of BSE-endemic countries. Article 3.8.4.2 of the OIE Terrestrial Animal Health Code provides a template for such subpopulations:[55]

- Clinical Suspects – cattle over 30 months of age displaying clinical signs consistent with BSE
- Casualty Slaughter – cattle over 30 months of age that are non-ambulatory; cattle over 30 months of age sent for emergency slaughter or condemened at ante-mortem inspection
- Fallen Stock – cattle found dead on-farm, or that die during transport
- Routine Slaughter – healthy cattle over 36 months of age

4. The targeted population, as described, is further confounded by the age of targeted animal varying with clinical signs or collection site. The targeted subpopulations should be confined to a single designated age.

5. To ensure that only animals within the targeted population are sampled, the targeted subpopulations should correlate to the "Primary Reason For Submission" section of the data collection form.

6. Prior to commencement of the enhanced surveillance program, the characteristics of the targeted population seem to have varied by state, and were largely divergent from those desired by enhanced surveillance, especially in regards to age. Conflicts with previous practices have served as a source of confusion allowing for sampling of animals not within the targeted population defined by the enhanced surveillance program.

7. Training materials that address the targeted population beyond that provided by the BSE Surveillance Manual and Plan are produced at the discretion of VS Area Offices or State Veterinarian Offices. To prevent geographical biases in sample data patterns, uniform outreach between states is needed and is best accomplished using national oversight.

8. The value of samples from animals <30 months of age with CNS signs is negligible compared to those >30months of age. These samples are allotted minimal points by the OIE code.[55] Animals <30 months of age are very unlikely to be affected with BSE since the incubation period averages 60 months,[23] and since diagnostic tests cannot detect disease during early incubation.[32] However, since BSE has been diagnosed in animals less than 30 months of age,[24, 25] the inclusion of these animals in the targeted population is

justified , but may be a relative misuse of resources.

9. The exception for FSIS allowing samples to be collected from antemortem condemns (including those without CNS signs) of any age, excluding veal calves, is not science-based. These animals should not comprise part of the targeted population since they are excluded from data analysis. The collection and testing of these samples is a waste of resources.

10. The second incisor may erupt between 26 and 38 months of age, varying with breed, nutrition, and climatic factors.[60] Therefore, animals with an erupted second incisor may not be >30 months of age, and animals without an erupted second incisor may be >30 months of age. In spite of the inaccuracy in dentition, it remains as the only practical, cost-effective, and internationally accepted means to assess cattle age when reliable animal records are lacking.

11. Animals that fit the targeted population are not necessarily condemned at antemortem inspection. Emaciated, injured, lame, or intermittently non-ambulatory ("walking downers") animals may pass antemortem inspection. Since the cause of lameness is often ambiguous, physical examination is inaccurate at differentiating primary neurologic from orthopedic disease. This avenue in which

animals at risk may evade surveillance is especially concerning from the aspect of food safety.

Sample size

Details: The size of the targeted population in the U.S. was estimated using the best current scientific knowledge of the U.S. cattle industry. On-farm mortalities of "adult" cattle were estimated to number 251,532,[61] "adult" cattle condemned (ante- and post-mortem) at slaughter with pertinent condemnation codes (including for emaciation, tetanus, CNS disorder, dead, injury, or moribund) were estimated to number 194,225,[62] and on-farm cattle with CNS signs were estimated to number 129 (based on foreign animal disease investigations). The estimated size of the target population, 445,886, is roughly 1% of the entire US cattle population, which is consistent with the range for the percent of the adult cattle population comprised of high-risk cattle reflected by European Union (E.U.) data.[59]

A detection limit (design prevalence) of 1 detectable case of BSE per 10,000,000 adult cattle, corresponds to 4.5 cases existing in the adult U.S. cattle population (size of 45 million adult cattle).[63] If all cases

would be found in the targeted (high-risk) population, a prevalence of $1.01E^{-6}$ (4.5/445,886) is expected within the targeted population. Using this prevalence with the sample size estimation method described by Cannon and Roe,[64] a minimal sample size of 268,500 was derived in order to detect one or more BSE cases at the 99% confidence level. To avoid spatial neglect and ensure basal representation of each the nation's regions this goal was geographically apportioned based on U.S. cattle population and distribution estimates (Fig. 2.1).[63] A census methodology was adopted, since the dependency of U.S. animal health surveillance systems on voluntary cooperation of selected sample collection sites precluded the use of a random sampling scheme. The goal of this approach is to collect as many samples as possible from animals of the targeted population over the 12-18 month enhanced surveillance period.

Several assumptions were made for the purposes of sample size estimation. As mentioned, the 4.5 potential cases of detectable BSE (1 per 10 million adult cattle population) are assumed to be restricted to the targeted ("high-risk") cattle population. This assumption was supported by the increased likelihood to detect BSE in a targeted population than in clinically normal cattle,[59] the low estimated risk of BSE in the US based on preventative mitigations,[51] and the fact that current diagnostic assays can detect disease no earlier than

approximately three months prior to the onset of clinical signs.[65] Second, the samples collected would be representative of the targeted population. Lastly, the diagnostic strategy, as implemented, has a high sensitivity and specificity.

Strengths:

1. Sample size has been determined with appropriate statistical justification, including a measure of overall sensitivity (design prevalence) and level of confidence. The level of sampling is acceptable.

2. A census-based sampling strategy is justified as an attempt to minimize the effect of nonrandom sampling.

3. The design prevalence of 1 detectable case of BSE per 10 million adult cattle is well below the estimated incidence of sporadic TSEs (i.e. 1 case of sporadic CJD per 1 million people),[66] and surpasses OIE requirements.[55] Such a conservative design prevalence errs on the side of over-sampling to increase the likelihood of detecting disease. This approach ensures maximal effort for disease detection.

4. The size of the targeted population may have been overestimated, since "adults" included in the NAHMS data were not necessarily

over 30 months of age, and since approximately 70% of the animals identified through FSIS data were postmortem condemns. Although cattle with these characteristics are not sampled by the surveillance program, their inclusion in the estimate of the size of the targeted population is a conservative decision, resulting in a larger targeted population, and therefore, a larger sample size to meet the specified design prevalence. This approach also errs in the favor of over-sampling to detect disease.

Areas for Consideration:

5. Although regional minimal sample goals have prevented geographical neglect, mechanisms cannot be established to ensure spatial bias is avoided. Therefore, data cannot be easily used to detect spatial trends in disease. Additionally, while nonuniform stratification of sampling will not reduce the ability of a surveillance system to detect disease, oversampling from certain geographical areas (with potentially reduced prevalence) complicates data analysis in the context of prevalence estimation. However, there is no statistical reason to believe that prevalence is different between the partitioned geographical areas.

6. Regional minimal sample goals were allocated based on where cattle reside, not where cattle are slaughtered or disposed of. Therefore, goals must be considered from the aspect of area where

cattle last resided in addition to area where samples were collected.

Case Definition

Details: The case definitions for BSE-affected cattle rely strictly on laboratory criteria.

Any bovid whose obex sample has histopathologic lesions specific for BSE, or characteristic immunoreactivity for PrP^{res}, demonstrated either by immunohistochemistry (IHC) or immunoblotting (WB), and confirmed by an OIE reference laboratory, is considered positive for BSE.

Additionally, the term "initial reactor" defines samples that are positive on the first screening test attempt. The term "inconclusive" designates such samples that are also positive when the screening test is subsequently repeated in duplicate.

Strengths:

1. Case definitions are simple and understandable; they are not confounded by clinical criteria. The methods used to verify positive cases are clearly described.

2. It is desirable that some case definitions (i.e., "inconclusive", "initial reactor") imply a level of certainty in the diagnosis. For example "inconclusive" or "initial reactor" imply that while samples are not negative for BSE, their potential to be positive is uncertain.

Areas for Consideration:

3. Case definitions do not provide a process for interpreting discrepant test results. This deficit allows potential for confusion or controversy regarding such test results.

4. The terminology adopted for ELISA-positive samples is misleading. Although politically advantageous, the term "inconclusive" underestimates the level of certainty in results. "Inconclusive" implies that results are debatable, uncertain, or not credible, whereas data suggest false positives rarely occur with the ELISA used by the surveillance program.[67] Terms such as "probable", "presumptive" or "suspect" are more appropriate.

5. The capacity of histopathological diagnosis of BSE needs to be more clearly defined by the case definitions. Histology should not be used alone to impart a diagnosis of BSE since perikaryonic vacuoles, indistinguishable from those seen with BSE, may be

incidentally found in certain brainstem nuclei of normal cattle.[68]

Sample Collection

Details: Beginning with sample collection, major surveillance steps are summarized in Figure 2.2. The enhanced surveillance program is dependent on participation of several types of collection sites:

- State or Federally inspected, or custom exempt (non-inspected) slaughter facilities – primarily those dealing in culled/aged cattle
- Salvage slaughter facilities ("3D/4D" – receiving dead, down, diseased, or disabled animals not for human consumption)
- Rendering facilities
- On-farm
- Veterinary diagnostic laboratories
- Public health laboratories (rabies-negative cattle)
- Other – including veterinary clinics, livestock markets, and contracted sample collection sites

State-formulated surveillance proposals identified predominate sample sources from facilities available to the state, enlisted collection sites,

and allocated sample collection indemnities. Participation by chosen sites is voluntary and is motivated by incentives which vary by region and include carcass disposal, carcass transport, storage fees, and sample collection fees. Sample collection fees are only provided to contractors, diagnostic laboratory personnel, plant employees, or accredited veterinarians; fees are not provided if federal or state employees collect samples. Incentives are only provided when sampling criteria have been met as defined by the targeted population. For most states, efforts were focused on inclusion of concentration points, facilities receiving many animals or carcasses befitting the targeted population. Therefore, most samples were expected to be derived from slaughter plants and rendering facilities. However, the cooperation of smaller sample sources was also recruited in the interest of identifying cattle with CNS signs. Slaughter facilities may contract sample collection from FSIS antemortem condemns to local salvage slaughter or rendering facilities; such arrangements must be approved by APHIS, as described in FSIS notices 33-04 and 29-04.[69, 70]

Samples may be collected by authorized federal or state personnel, accredited veterinarians, or APHIS-contracted, diagnostic lab, or plant employees. Identification of the appropriate animals for sampling requires that the sample collector, and those involved in the receipt of cattle at participating facilities, have a firm understanding of the

targeted population. Additionally, reporting of animals for surveillance demands a similar understanding of cattle producers. Training materials which described the targeted population and aging of cattle by dentition were distributed to collection site workers.

For cases in which cattle are presented to sample collection facilities alive, animal euthanasia is not addressed by the BSE Surveillance Guide.

Brainstem samples are collected through the foramen magnum, using a brain spoon, after removal of the head. An appropriate brainstem sample includes obex, and is affected with little contamination or postmortem decomposition. Brainstems are individually packaged in plastic tubes that are labeled with a unique BSE sample identification bar code supplied by the USDA. Sample collectors were instructed on sample collection technique by NVSL personnel, or APHIS or state personnel who were trained by NVSL. Additionally, training materials produced by APHIS or state-based initiatives were provided to collection site workers.

Animal identification items (drawings or digital pictures of brands, removed tattooed hide, ear tags, etc...) are collected from each animal sampled, bagged, labeled with the sample number, attached to a copy of

the USDA BSE Surveillance Submission Form and saved by the sample collector until negative results are received.

Samples are enclosed with cool packs in insulated packages, and are shipped by overnight contract delivery service (FedEx), same-day courier service, or by hand delivery. A paper copy of each submission form must be submitted to the diagnostic laboratory with the samples. The sample collector notifies the diagnostic laboratory by facsimile, telephone, or e-mail, of tracking information, the number of incoming samples, and referral number. Delivery verification and trouble-shooting is the responsibility of the sample collector.

Carcasses from negative animals are disposed of in compliance with Federal, State, and local laws. Carcasses and offal from "inconclusive" or positive animals may be disposed of by one of the following: rendering for non-animal feed use by dedicated facilities, burial in a landfill, burial on-farm, alkaline digestion, or incineration. Rendering facilities may refrigerate or freeze carcasses, or may proceed with rendering and hold batches of final products, pending test results. Should a positive animal occur with the latter method, an indemnity would be supplied for the disposed batches of products.

Strengths:

1. Tissue sampling methods are thoroughly detailed and depicted in

the BSE Surveillance Guide and other training materials. Training efforts for sample collection technique seem to have been effective. This is evidenced by the relatively few samples which were collected, but not tested, totaling 5,314 (0.95%) as of December, 2005.

2. A variety of data sources have been enlisted by the surveillance program. All major avenues for potential sample collection from animals befitting the targeted population have been recruited. For example, the inclusion of public health laboratories is quite insightful.

3. Chosen incentives seem to have been effective in obtaining the cooperation of selected collection sites.

4. The number of collection sites participating in the surveillance program has sufficed to exceed the large sample size goal.

5. Sample handling protocols are described in depth, and based on field observations, seem to be rarely deviated from.

Areas for

6. The only trigger for sample and data collection is collector-

Consideration: dependent recognition of an animal that is relevant to the targeted population. This emphasizes the need for clarity in training materials that address the targeted population (refer to "Targeted Population" sections 3, 4 and 7). The capture of all appropriate high-risk animals is necessary to improve the validity of the surveillance program.

7. Sampling is not random since participation in the surveillance program is noncompulsory. Although a census-based sampling strategy somewhat reduces the effect of nonrandom sampling, there are no regulatory or legal requirements to report cattle fitting the targeted population. Therefore, samples are biased to be collected only from those who benefit from their submission. Incentives for collection site participation vary by geographical area and there is uneven enlistment of various types of collections sites by geographical area. Thus, there is not an equal chance of samples to be derived from each type of collection site. This is in addition to the effect of inherent variability in the likelihood or degree of an individual collection site's voluntary participation. Furthermore, there is differential representation of targeted subpopulations by collection site. As a result, animals are erratically represented on the basis of age, condition, and geographical location. This is a detriment to the representativeness of the system and is reflected

by the variation in average sample value (surveillance points per sample) among geographical regions (see Table 2).

8. A disincentive for producer participation exists that may bias collection of samples away from animals with disease suspicious for BSE. Producer compliance is discouraged by the considerable and uncertain financial consequences that may ensue from detection of a positive animal. Additionally, financial recompense for surveillance participation to encourage motivation is insufficient, consisting of variable carcass disposal and transport fees as allotted by state-based surveillance proposals. Since the enhanced surveillance program depends on voluntary contribution, and since submission of carcasses to disposal facilities involved in sample collection is optional in this country, willingness of producers to cooperate is essential. Furthermore, without producer participation, animals within the targeted population that are remote from disposal facilities involved in sample collection will be largely neglected by the enhanced surveillance system.

9. The positioning of selected sample collection sites probably does not evenly represent the geographic distribution of the targeted population. Collection sites seemed to have been selected based on willingness or ability to participate, not necessarily on

representativeness. The degree to which these collection sites represent the cattle population of their respective region, based on the geographical area which they service, has not been evaluated. This problem especially concerns collection sites receiving fallen stock, and emphasizes the fact that targeted sampling strategies often detract from representativeness. To improve representation of areas remote from collection sites, enlistment of accredited veterinarians for on-farm sample collection is essential.

10. There are no checkpoints in place to ensure that only samples derived from the targeted population are tested, or that compensation is only provided for targeted samples.

11. Contracts for collection of samples from FSIS antemortem condemns off-site (not at the slaughter facility where the animal was condemned) facilitates sample data inaccuracy or loss. Condemnation tags are removed prior to animal transport, in accordance with FSIS Notice 40-04,[71] and limited animal information may be provided to the rendering facility. This practice facilitates the loss of sample data including condemnation number (Z-number), condemnation code, clinical signs, and even the fact that the animal was condemned. The loss of antemortem condemns from the data is apparent when comparing the numbers

of condemned animals (provided by FSIS data) and the number of samples recorded as originating from condemned animals.

12. Animals that arrive in poor condition for slaughter (dead or down) may be refused to be unloaded onto the premises.[70] Slaughter facilities have no interest in accepting animals that they must pay to have disposed. These animals will go untested unless there is a disposal facility in close proximity the producer chooses to use. The potential for this problem has been recognized, and the importance that the USDA take steps to assure that targeted animals excluded from slaughter for the purposes of food safety are not systematically discarded from surveillance has been emphasized.[56]

13. The validity of data obtained from samples collected by parties receiving financial compensation is questionable. Essentially, these facilities are motivated to collect maximum sample quantities, and may neglect various animal characteristics (especially age) to improve profits. There is no established oversight to ensure samples are only collected from animals within the targeted population and that sample data is accurately recorded.

14. A disincentive exists for the enlistment of slaughter and disposal-

type facilities, in that such facilities are apprehensive about their clients becoming aware of their involvement in BSE surveillance. Should a potential client wish to evade surveillance, the services of a competing facility, not involved in surveillance, may be elected. It is unclear how involvement in the surveillance program has affected participating businesses. Additionally, clients may be reluctant to purchase animal by-products from disposal-type facilities participating in surveillance when considering the repercussions of such product being contaminated with material derived from an animal that tested positive for BSE.

15. The collection and temporary storage of animal identification items are not implemented in the field.

16. Sampling methods fostered by the surveillance system do not consider data sensitivity and confidentiality issues. Assurance of confidentiality regarding test results may improve participation in the program (refer to "Sample Collection" sections 8 and 14).

17. Targeted animals that are slaughtered or condemned at postmortem inspection are not sampled. For example, intermittently nonambulatory animals are part of the targeted population, but are not condemned at antemortem inspection, and thus, will not be

sampled for surveillance. This provides an avenue where targeted animals, especially those with subtle clinical signs, may evade surveillance. Antemortem inspection only condemns animals with definitive clinical signs that clearly implicate a condition such as CNS disease, fever, or invasive cancer.

Data Collection Forms

Details: The USDA BSE Surveillance Submission Form and USDA BSE Surveillance Data Collection Form are completed by the sample collector either by hand (paper forms) or electronically, through the National Animal Health Laboratory Network (NAHLN) website (nahln.aphis.usda.gov/nahln/jsp/login.jsp). In either case, a hard copy of the BSE Surveillance Submission Form must be submitted to the diagnostic laboratory such that samples can be appropriately identified and assigned an accession number, and results can be supplied to the submitter. Samples that are not accompanied by the appropriate submission forms with all necessary information are not tested. Some states demand the collection site or diagnostic laboratory forward submission forms to the Area Veterinarian in Charge (AVIC) office.

All sample data must eventually be entered into the NAHLN website, the interface for the centralized database where surveillance data, including test results, are stored. If the collection site is incapable of completing data entry electronically (completed paper forms only), a hard copy must also be forwarded to a USDA:VS office. Data form trafficking is summarized in Figure 2.3.

The USDA BSE Surveillance Submission Form must be completed for each sample batch for a single collection site and date. This form requests submitter information including collection site type, collection site address, and collector identification. For samples from antemortem condemns that were collected by a contractor, there is an additional section for slaughter site information. Additionally, a BSE sample ID bar code, duplicate to those on the sample tubes, is supplied on this form for each sample.

The USDA BSE Surveillance Data Collection Form must be completed for each animal sampled. Several parameters are recorded on this form. The sample collector must provide a "Primary Reason for Submission", used for analysis of surveillance streams, by selecting from the following:

- Highly suspicious for BSE (as described in VS Memo 580.16)
- Nonambulatory/disabled (Downer)

- Dead
- CNS signs
- Other clinical signs that may be associated with BSE
- Rabies suspect
- FSIS – Antemortem condemned at slaughter
- Apparently healthy adult at slaughter

A section for owner (animal source) address and contact information must be completed. This is followed by a section of animal information including sex with neuter status, age, verification that the 2nd incisor is erupted, and country of origin if non-domestic. The collector is asked to provide all types of ID the animal has; several types are requested including silver tag#, owner ear tag#, vaccination tag#, condemnation tag#, back tag#, bangle tag#, ear tattoo, brands, microchip, and other. Finally, the collector must select the most appropriate animal characteristics from lengthy lists of breeds, clinical signs, and FSIS condemn codes (if applicable).

Both forms require a referral number, a unique identifier that is used to associate the Submission Form with the Data Collection Form. The format consists of 12 alpha numeric characters. The first two characters are the State's abbreviation, the second three are the collectors initials, the following six are the collection date, and the last character is a letter

representing designating number of submission batches (A – first, B – second). For FSIS collections, the state abbreviation is replaced by the FSIS Establishment Number.

To ensure and monitor data entry accuracy, VS randomly verifies that electronically recorded sample data accurately represent those provided by corresponding submission forms. These "internal audits" are performed periodically (roughly biannually) and consist of examination of approximately 50 randomly selected sample forms that are representative of all states. The data on these forms are compared to the corresponding data stored in the NAHLN database.

Strengths:

1. The data recorded by the submission forms are detailed and thorough. There is sufficient animal and herd information recorded such that a positive animal can be traced with optimal precision, although limited by the shortcomings of the present cattle identification system. No demographic parameters that are required for data analysis using the OIE Code, or the BSurvE model, have been excluded. The recorded parameters suffice to provide meaningful and relevant surveillance reports.

2. The NAHLN interface is user-friendly and simplifies the data-entry process. It facilitates collection of accurate data by maintaining

safeguards against entry of contradictory or nonsensical data.

3. The information recorded on the submission forms accurately associates the collected data to the corresponding tissue sample.

Areas for Consideration:

4. Quality of training and supervision of persons who complete surveillance forms could be improved. Since there is little oversight and deterrent for improper form completion, the validity of data is questionable. Perhaps including a cautionary statement, that implies monetary compensation will not be supplied for samples with inaccurate or incomplete data, should be included somewhere on the forms.

5. Submission of data collection forms to multiple parties is laborious for the sample collectors, and is a waste of resources. Only form submission to diagnostic laboratories, and to VS offices for data entry (if necessary), seem mandatory. Sample information and testing results can be obtained from other parties through the NAHLN website.

6. The trafficking of submission forms is not uniform. This detracts from simplicity of the surveillance system and is a hindrance to structured oversight. The task of form forwarding to a second

party is unusual for diagnostic labs; reliance on this mode of trafficking provides an avenue for potential data loss.

7. The logistics of some collection sites preclude the same person involved in sample collection complete the submission forms. For example, samples from salvage slaughter facilities may be collected by workers involved in the slaughter process, whereas the forms for the samples are completed by a separate person in an office. Under such circumstances, the only information about the sampled animal available to those completing the forms is provided in paperwork with which the animal was submitted. This information is not verified by examination of the sampled animal and often does not include clinical signs, additional identification, age, sex, or breed. Therefore, these parameters must be habitually completed based on the most likely or plausible characteristics. For example, most animals from dairy farms received by rendering facilities are dead, adult, female, Holsteins; data for animals recognized as being from a dairy farm would be assigned as such.

8. The complexity and poor clarity of the supplemental data form is a deterrent to its proper completion, resulting in poor sample data accuracy. The system should pursue fewer, quality data rather than excessive, inferior data. Greater than 50 percent of the time

allotted for sample collection is spent on form completion. Most of the time spent on form completion is dedicated to recording detailed owner information. Since this information is usually not submitted with animals for slaughter/disposal, to adequately complete this section, many facilities must look up the information in the phone book using the owner's name and city or zip code. Several other requested parameters seem superfluous. In most rendering and salvage slaughter operations, only heads of the animals are presented for sample collection. Sex is determined based on the size of the head; it seems unlikely that an animal could be determined as neutered using this technique. Many bovine breeds cannot be accurately distinguished based on morphologic features, especially when only the head is examined. Additionally, this sample data has little usefulness beyond determining animals as beef versus dairy breed. The hide typically cannot be examined for brands or tattoos. Even if this was possible, these forms of identification are usually not legible.

9. Data derived from the "Primary Reason for Submission" section of the USDA BSE Surveillance Data Collection Form is used to determine and report trends in surveillance streams, and is used in conjunction with cattle age to determine whether samples are "targeted" or "non-targeted". As categorized, the data derived here

require extensive validation efforts and have little reliability and therefore usefulness (refer to "Data Analysis and Dissemination). Many of the categories are redundant with parameters recorded elsewhere, such as the clinical signs section. Categories overlap and are non-discrete. Those completing the forms are forced to choose between multiple categories that seem appropriate. The most desirable choice from the aspect of data analysis is not clearly apparent to the person completing the form. For example, FSIS condemns may be "dead", "nonambulatory/disabled", or have "CNS signs", each of which is a separate category. The difference between "CNS signs" and "Highly suspicious for BSE" may not be apparent unless those completing the form have read VS Memo 580.16. The category "other clinical signs that may be associated with BSE" requires a veterinary skill level that is likely beyond the average sample collector. Furthermore, this section could additionally assist sample collectors in differentiating animals that should be included in surveillance if it were better correlated to the defined targeted population.

10. The accuracy of the clinical signs data is questionable since completion of this parameter does not require veterinary expertise, and since information pertaining to clinical signs and history is often passed by word-of-mouth and cannot be verified by the

sample collector. Without veterinary skill, the meaning and significance of certain clinical signs provided by the form may not be apparent.

11. The clinical signs data are used to allocate samples into the surveillance streams provided by the OIE Code and BSurvE model, both of which are used for analysis of the Enhanced BSE Surveillance results.[72] Specifically, the clinical sign profile is used to designate samples as "clinical suspects". This requires estimation of the specificity of certain clinical signs for BSE, based off findings from the UK BSE epidemic.[73] However, this task was complicated by incongruence in the clinical signs recorded by the present surveillance data forms and the clinical signs evaluated by Wilesmith et al. To improve the accuracy with which animals are designated "clinical suspects", perhaps the clinical signs provided on the data collection forms should be formulated to more closely represent those that have known specificity for BSE. For example observations such as parlor kicking, fetlock knuckling, temperament changes, nervous entrance, abnormal ear carriage, and head shyness should be included in the forms whereas exopthalmia should be excluded. Futhermore, certain observations could be collapsed to a single parameter (i.e. "ataxia" and "abnormal gait" could be collapsed to "ataxic").

12. Since the ability of the average lay person to accurately record sample information using a computer interface is unknown, consideration should be given to establishing qualification and training requirements for those involved in data entry.

13. Results of "internal audits" indicate that data forms average 2 non-completed parameters per form. Additionally, discrepancies between raw and electronic data range from 1.2-8.3 discrepancies per form.

14. The FSIS Condemnation Code section includes codes that are not conventionally used for antemortem condemnation, such as pneumonia, metritis, etc... The most important codes include: emaciation, tetanus, CNS disorder, dead, injury, moribund, nonambulatory, and rabies. This leaves several additional codes which detract from selecting codes pertinent to surveillance. Some codes are nonspecific and provide little information (i.e. general misc.). In addition, there is overlap and redundancy between many codes. The process of code selection is highly operator dependent, as rules which designate proper code choice seem to be lacking. Therefore, the value of the condemnation code data (beyond the fact the animal was condemned) is questionable, especially if data

which more specifically characterize the sample can be found elsewhere (i.e. clinical signs section).

Diagnostic Strategy

Details: Several state veterinary diagnostic laboratories were contracted by the National Veterinary Services Laboratory (NVSL) to perform rapid screening tests for BSE diagnosis. Laboratories were selected based on regional sample allocations, presence of other TSE's in the laboratory's region, and prior experience with TSE diagnosis. Laboratories must pass a proficiency test prior to operation, and then consequently at six month intervals to maintain contracted status. Additional laboratory quality assurance consists of annual inspections by NVSL personnel, annual maintenance inspection from BioRad personnel, and compliance with integrated weekly and monthly quality checks listed in equipment manuals. Proficiency testing is not required for NVSL pathologists involved in immunohistochemistry (IHC) interpretation.

Although the selection of screening test was left to the discretion of the diagnostic laboratory, all participatory diagnostic laboratories use

BioRad TeSeE with high-throughput automated equipment. Other tests licensed by the Center for Veterinary Biologics (CVB) include IDEXX HerdChek BSE Antigen EIA, ENFER, Prionics Check Western Blot, and Prionics LIA. CVB standards require kit satisfactory sensitivity, specificity, and ruggedness (reproducibility in other laboratories), production in a federally inspected facility, and approval to any change in production protocol.

Samples are sent to a designated diagnostic laboratory determined by collection site location and diagnostic laboratory sample capacity (see Figure 2.4). Although adherence to this scheme results in optimal testing efficiency and is encouraged, collection sites are not prohibited from submitting samples to the diagnostic lab of their choice. For regions in which samples cannot be delivered to the testing site within 24 hours, a screening test is not used. Therefore, brainstem samples from Alaska, Hawaii, and Puerto Rico are formalin-fixed and shipped to NVSL for IHC.

Diagnostic laboratories trim the appropriate subgross anatomic site from the obex sample and prepare a tissue homogenate for the screening test. The remaining brainstem sample and surplus homogenate are stored at a low temperature for 5 days. If a known technician error occurs during testing, the test cannot be completed and

results are not recorded. The mistake is recorded following independent laboratory-specific quality assurance initiatives, and the test is restarted.

Testing and reporting strategy is summarized in Figure 2.5. The following are potential screening test results:

- "not detected" – negative
- "not detected, not obex" – negative; unable to confirm proper sample location
- "no test" – not tested at discretion of laboratory technician
- "initial reactor" – positive on first test
- "inconclusive" – positive when repeated in duplicate

The cut-off value for optical density (OD) that indicates a positive sample is dictated by NVSL protocol; the cut-off is conservative (erring for a false-positive) in that it is slightly lower than that recommended by BioRad. The BioRad software additionally subcategorizes results as "NVSL 1" (optical density value is positive) or "NVSL 2" (optical density value is in "grey zone", below the cutoff for positive samples); both are considered positive. For "initial reactors", the remaining tissue homogenate is used for two subsequent ELISA's ran in parallel. If one of the repeated ELISA's are positive, the sample is considered "inconclusive"; otherwise the sample is considered negative. The

brainstem and any remaining homogenate from which "inconclusive" results are derived, are forwarded overnight to NVSL for confirmatory testing by IHC. The objective of this testing strategy is to minimize false positive results derived from technician error and is recommended by manufacturer instructions. At NVSL, one section of formalin-fixed, paraffin-embedded obex is prepared for immunohistochemical evaluation by a pathologist. Samples that contain specific PrP immunoreactivity patterns are presumptively positive and are hand-carried by an NVSL pathologist to an OIE Reference Laboratory for confirmation.

"Not detected, not obex" samples comprise those in which the appropriate sub-gross anatomic site for testing could not be reliably identified. These samples appear to be brain stem, but obex can not be identified. Such samples may have advanced post-mortem tissue decomposition, or may be physically disrupted beyond recognition (from gun shot or poor collection technique). "No test" samples may be discarded at the discretion of the laboratory technician for similar reasons, but are usually samples that cannot be recognized as brain stem (ex: spinal cord, cerebellum). Collection fees are not relinquished for samples that remain untested for any reason.

NVSL is responsible for oversight of contracted laboratories. Detailed

standard operating procedures (SOPs) exist to address training of those performing the assay, diagnostic assay protocol, quality assurance measures, and the process of reporting results. These are promptly updated whenever changes are made.

Strengths:

1. The uniformity in procedure between laboratories is advantageous to the simplicity of the surveillance system.

2. The oversight of the NVSL is diligent. SOPs address all pertinent responsibilities of the contracted laboratories. Changes in protocol are documented in a timely fashion.

3. Quality assurance measures are complete and methodical.

4. Turn-around time is fast for screening and confirmatory test results, usually occurring within 24-48 hours, or 5-7 days from time of sample collection, respectively. Including IHC, the mean days between sample collection date and data entry of test result is 5.6 (mode 2.0), and the mean days between initial data entry of sample data and entry of test result for samples is 2.5 for samples collected between June 1, 2004 and January 2, 2006. The interval between sample collection and results entry may be relatively delayed by collection sites which collect samples daily, but only ship samples

for testing a few times per week.

5. The decentralization of laboratories conducting screening tests, and the number of laboratories recruited for involvement in BSE surveillance, has minimized turn-around time for test results.

6. Adoption of a rapid immunodiagnostic screening test has also significantly minimized turn-around time for test results. The selected ELISA has been validated by the European Commission and is used for BSE surveillance in several other countries.[67]

7. All participating diagnostic laboratories are accredited by the American Association of Veterinary Laboratory Diagnosticians. Accreditation requires that laboratories conform to guidelines for personnel qualifications, facility specifications, and diagnostic technique standards. Thus, diagnostic tests performed by these laboratories are considered to be of highest quality.

8. The use of an ELISA for screening, and IHC for confirmation, is consistent with international expectations.[30, 56] The adoption of methods equivalent to those used by surveillance programs conducted by other countries facilitates the comparison of surveillance results.

Areas for Consideration:

9. The dependency of the surveillance system on a single screening diagnostic assay is disconcerting. Conservation of testing procedure is desirable from the aspect of simplicity, quality assurance and oversight. However, the function of the surveillance system is essentially vulnerable to problems with production or distribution of the diagnostic assay kits. Furthermore, the use of an additional screening test would allow comparisons in test data trends that may provide insight to the performance of the primary diagnostic assay used by our system. The hesitation by diagnostic laboratories to adopt a test other than the BioRad ELISA is understandable, given the uncertainties associated with fostering an assay with which American diagnosticians have little experience.

10. In diseases of low prevalence, the positive predictive value, that is the percent of patients with positive test having disease, is low. Therefore, relatively high numbers of false positive test results are expected; positive test results are less reliable. Surprisingly, false positives ("inconclusives" or "initial reactors") encountered by the enhanced surveillance system are exceptionally rare. In the European Commission study that validated the BioRad ELISA (see Test D, CEA, France),[67] the assay correctly categorized 1000 negative samples as negative, thus earning a specificity of 100% (95% confidence limit one-sided Poisson, specificity of 99.7%).

Assuming a true specificity of 99.9%, using a binomial distribution, the number of false positives should be considerably higher ([1-.999] * 556,604 = 556 expected false positives, as of December, 2005). There is no evidence that the sensitivity of the present testing strategy is poor, however, there is no evidence to defend its adequacy. Since negative samples are not re-tested, the number of false negatives is unknown. An alternate screening test is not available to the surveillance system to serve as a comparative source for evaluating the BioRad ELISA performance. Also, the false positive rate has not been compared with that of other countries using the BioRad ELISA. Therefore, the sensitivity of our testing strategy cannot be estimated and testing strategy should be adopted so as to enhance confidence in negative test results. Such measures could include testing a random sample in parallel with a second screening test, or addition of a tissue-based positive control (i.e. aliquots of CWD or Scrapie positive brainstem material) to each test run. The testing strategy may be more specific than anticipated due to the use of automated equipment that was not used by the validation studies and that may reduce certain technician errors. Another possibility is the lack of "splash effect", where material from positive samples splashes into adjacent sample wells, contaminating negative samples and resulting in false positives. This type of error may be significantly

reduced in testing populations of low disease prevalence.

11. Consideration could be given to implementing a more rigorous screening modality for samples from clinical suspects. Performing two diagnostic tests in parallel on such samples would increase the sensitivity of the testing strategy and would increase the confidence in negative test results. Additional tests that could be considered include histology, IHC, WB, or an additional ELISA. Such an approach would seem to benefit the present system since negative samples are never re-tested, and consequently the level of confidence in negative test results is undetermined and relies on results derived from BioRad TeSeE validation studies. In addition, countries experienced in BSE diagnosis use diagnostic assays held in higher regard than the ELISA (i.e. histology, IHC, or WB) to screen tissues from clinical suspects;[74] thus the U.S. may be criticized screening clinical suspects with an ELISA.

12. Exclusion of decomposed animals produces bias against sampling animals that are remote from collection sites or reside in warm climates. Additionally, tissue decomposition provides an avenue in which targeted animals can escape surveillance. However, the exclusion of such samples is practical, since there is little value in a negative test result from a sample in which the obex is not

identifiable. Although value in a positive test result remains, these samples would prove difficult to confirm. If samples have reached liquid-state, confirmation by immunohistochemistry is not possible. Even if a confirmatory test can be performed, the inability to recognize the appropriate location for testing may produce results that are discrepant with the screening test. Regardless, given the value of samples from clinical suspects, perhaps samples from these animals should be tested regardless of tissue quality.

13. Collection sites should be encouraged to collect every sample possible that fits the targeted population, regardless of sample quality. At present, collections sites variably collect samples from animals with potentially decomposed brainstems as they will not receive financial compensation for untested samples. If decomposition is questionable, most collection site workers are unable to determine if the sample is too decomposed to test and error on the side of not-testing. If there is evidence of exterior decomposition (sloughing of skin from the hide for example), the potential sample is often not even examined (i.e. by exposing the foramen magnum to evaluate brainstem integrity). Sample testability should always be determined by laboratory technicians. Collection fees should not be refused for an individually decomposed sample, however, refusal of fees seems reasonable for

the event of post-collection decomposition that affects entire accessions of samples (for example, batches of samples that were not properly shipped to the diagnostic lab).

14. In regards to "initial reactor" samples, testing in series to confirm ELISA results may not be a desirable strategy for a screening test since this approach in effect decreases the sensitivity of the testing scheme. However, this approach was used for the validation studies performed by the European Commission which determined the BioRad TeSeE assay to have 100% sensitivity.[67] Therefore, using this strategy will have no effect on expected performance. Additionally, it is very unlikely that this approach will affect the ability of the surveillance to detect true positive animals, as the likelihood of a true positive test reverting to a negative on two subsequent tests is negligible.

15. Diagnostic strategy could be improved by using the WB in parallel with IHC for confirmation of "inconclusive" samples. Controversy exists as to whether IHC or the WB should be considered the "gold standard" for TSE diagnosis. WB is equally sensitive as IHC, and WB has the potential to evaluate a larger portion of tissue for PrP^{res} than IHC.[75] The WB results are timelier since a formalin-fixation step is not required. Information that cannot be derived from IHC,

and that may provide some insight to the ELISA results, can be derived from immunoblotting. For example, certain band patterns of the WB can indicate poor tissue homogenation and digestion, each of which are capable of causing false positives on the ELISA. Additionally, the WB method elucidates molecular characteristics of PrP^{res}, such as glycoform pattern or electrophoretic mobility, that may be useful in differentiation of TSEs (i.e. BSE from CWD),[76] or recognition of atypical BSE.[44] Such information cannot be derived from IHC results. Since the WB demands considerable time, personnel, equipment overhead, and technical expertise for reliable interpretation, NVSL has been hesitant to adopt this modality to supplement IHC results. Only tissue from IHC-positive cases is forwarded to an OIE reference lab for confirmation, through use of WB among other possibilities. To increase confidence in negative results, similar protocol for "inconclusive" samples is desirable. However, the potential consequences of disagreement between test results obtained from separate laboratories must be considered. This is especially possible when considering the issue of sharing limited tissue, and the potential for discrepant results simply due to sample partitioning artifact.

16. The use of histology for confirmation of "inconclusive" samples

should be considered. Histology is more cost-effective than IHC. Furthermore, the presence of spongiform change in the grey matter of specific neuroanatomical sites will increase the confidence that false positive results have not occurred with tests which detect PrP^{res}. However, freeze artifact associated with tissue storage may obscure microscopic lesions, and samples affected with autolysis may not be suitable histopathologic diagnosis. Additionally, the lack of microscopic lesions does not exclude a diagnosis of BSE. Countries experienced in BSE diagnosis use histology as the primary test for clinical suspects, and as a confirmatory test for ELISA positive samples from fallen stock or casualty slaughter; if histology is negative or inconclusive, then IHC and/or WB is used.[74]

17. The independent maintenance of records that address laboratory error by individual laboratories seems suboptimal since little can be done with the recorded information. As an internal problem, records may best be left to the laboratory quality assurance system. However, management by a central oversight system is desirable to ensure organization and accessibility, and so that trends in error can be identified and preventative measures can be pursued. Additionally, these records may provide insight to account for the deficiency in false positives. Such records should be standardized

(conserved between laboratories) to include why and from what stage the test was restarted (brain, digestion, etc...).

18. Testing of a single neuroanatomical site may neglect the recognition of variant spongiform encephalopathies, such as BASE, which has atypical distribution of PrP^{res} accumulation, tending not to involve the dorsal motor nucleus of the vagus nerve.[44]

19. "Initial reactor" and "inconclusive" samples should be permanently recorded as such in the database, in addition to final (confirmatory) test results. Since only three of the four "inconclusive" samples can be found in the database, as of December, 2005, it is apparent that screening test results may be replaced by confirmatory test results. This provides an avenue for loss of valuable data. For example, the value for the number of "initial reactor" samples is needed to estimate the positive predictive value of the testing strategy.

20. SOPs should be readily available to the public. Transparency innately and effectively improves confidence of consumers and trading-partners in the validity of the surveillance program.

21. Records of quality assurance measures, including results of proficiency testing, should be maintained in a centralized database. These records should be managed by a nonbiased third party that is not involved in test administration or completion, such that they are readily accessible and their validity is not questionable.

Reporting of Test Results

Details: Most diagnostic laboratories test samples within 24 hours of receipt, except on Sundays. Accessions for which samples are received by noon usually receive results on the same day. Reporting of test results is limited by data entry, in that results can not be recorded by the diagnostic laboratory until the sample information has been entered to the NALHN database.

Testing results are available to qualified personnel on the NAHLN website. The day of screening test completion, results are entered into the NAHLN database and the submitter and AVIC are notified by the diagnostic laboratory. In some states, the state veterinarian is additionally notified. Sometimes, results must be reported to more than

one submitter. For example, results from contractor-collected samples from antemortem condemns must be reported to the contracted collector and FSIS. For "inconclusive" results, NVSL is notified. NVSL notifies the AVIC and submitter after 2:30 ET (after commodities close). The submitter and AVIC are notified by NVSL of negative IHC results. Following positive IHC results, the USDA:APHIS BSE Response Plan and the FDA Response Plan are initiated (see below).

Strengths:

1. Laboratory turnaround time is fast (refer to "Diagnostic Strategy" sections 4-6) and, based on field observations, results are usually entered into the database within 12 hours of test completion.

2. Results are readily available to any authorized party of interest through the NALHN website.

Areas for Consideration:

3. Since "Not Detected, Not Obex" samples comprise 6,228 (1.1%) of all tested samples (as of December, 2005), and since these samples are excluded from data analysis, it is necessary to understand the reasons for which this waste of resources occurs. Reporting results as "Not Detected, Not Obex", or "Not Tested" is non-descript. There seems to be overlap in the criteria for which a sample may be untested, or tested, but designated as "Not Detected, Not Obex". The reason why a sample is "Not Obex", including wrong anatomic location, decomposed, or physically disrupted tissue, is not

recorded. Similarly, specific reasons for a sample going untested are not recorded in the database. The rationale for disregarding collected samples from testing, and specific reasons for which a sample is of poor quality, should be reflected by the database. Such methods would allow identification of surveillance streams that are more likely to produce poor-quality samples. These data are valuable for measuring biases derived from exclusion of decomposed samples, improving fiscal efficiency of the surveillance system, and implementing policies to improve sample quality. These data seem vital to ensure high-risk animals are not systematically discarded by the system. Finally, more descriptive results are a more meaningful source of correctional feedback for poor sample collection practices, that would occur independently of the voluntary action by the diagnostic lab or AVIC which is presently required.

4. The requisite that diagnostic laboratories report test results to the sample collector, an AVIC office, and possibly even a secondary party involved in submission (i.e. FSIS) is excessive, unreasonable, and inefficient, especially considering that results are available to interested and qualified parties through the NAHLN website. Such practices represent wasted resources and disrupt laboratory function, possibly prolonging turnaround time for tests underway.

5. Reporting of results should not be hindered by data entry. That is, given that a sample ID and reference number have been provided, diagnostic laboratories should be able to record results on the NAHLN network.

Communication Pathways and Feedback Mechanisms

Details: Few communication pathways are succinctly suggested in the Enhanced BSE Surveillance Plan. First, as discussed earlier, collection sites contact the respective diagnostic laboratory to ensure that personnel are aware of incoming samples and shipping information. Second, NVSL and contracted laboratories have been designated to provide feedback to the VS Area Offices regarding sample and sample data quality. If deficiencies are reported, the surveillance plan designates the VS Area Office as the responsible party to pursue corrective action.

When hard-copy forms are missing essential parameters, VS personnel involved in data entry must contact the collection site in attempt to reconcile the missing data as accurately as possible.

Communication pathways involved in reporting of results have been described above.

Protocol does not exist to document changes in policy and to notify those implementing surveillance of such. Surveillance adjustments or clarifications have been in the form of various VS or FSIS memos. Sample collection sites may only be notified of changes by word of mouth, at the discretion of VS Area Offices.

Strengths:

1. Since the simplicity of the surveillance program is high, the effect imparted by deficiency in communication pathways seems to have been slight. Perhaps due to the conscientious intervention of VS Area Offices, the surveillance process has maintained order despite a general lack of structured communication means.

Areas for Consideration:

2. Deficiency in protocol for feedback mechanisms leads to low efficiency of the surveillance program. Deficiency in documentation of changes in policy confounds the poorly established communication pathways.

3. There is deficient compliance with feedback mechanisms that have been defined by the surveillance plan. For example, based on field observations, collection sites inconsistently inform the respective

diagnostic laboratory of incoming samples and shipping information.

4. The surveillance plan designates diagnostic lab personnel to provide feedback regarding sample quality and sample data quality, however, this occurs inconsistently as implemented. Laboratory technicians do not review the thoroughness and accuracy to with which forms are completed, beyond data needed for sample identification. When samples receive the results, "not tested", or "not detected, not obex", sample collectors are often corrected at the voluntary discretion of the VS Area Office. Since laboratory compliance with this responsibility is suboptimal and nonuniform, assignment of these responsibilities to the VS Area Office should be considered.

Data Analysis and Dissemination

Details: Weekly reports are produced by VS which provide test result counts, including number of negative, inconclusive, positive, and total samples. Additionally, these provide the results of confirmatory IHC for

inconclusive samples. These are available to the general public online at the USDA's BSE Test Results webpage (www.aphis.usda.gov/lpa/issues/bse_testing/test_results.html). In addition to these data, the VS National Office also receives tallies of samples by state of collection, or state of animal origin, with comparisons to projected sample goals. "Unobserved" reports, which tally and describe samples which are missing test results, are generated by VS daily for diagnostic laboratories, and weekly for the VS National Office. Daily reports produced by VS total new samples by state of collection and state of animal origin; these are distributed to VS Area Offices. Lastly, VS generates weekly reports listing accessions that have potential to be duplicates for VS Area Offices.

The NSU is the primary responsible party for data analysis. Several areas of sample data required correction. Some of these areas developed as a result of change to the data collection form and database format after the first five months of surveillance. However, many occurred due to inconsistencies in the manner in which forms are completed. A systematic and random methodology could not be adopted to recognize errors in data entry, therefore identification of problem areas relied upon review of weekly BSE reports for data that appeared erroneous. Records for approximately 7,200 samples, recognized to have potential inaccuracies, were verified or corrected by

VS area offices. Measures were taken to reconcile data that were affected with one of the following problems:

- The "Primary Reason for Submission" was missing.
- The "Primary Reason for Submission" for samples that had Z-tag identification numbers, or condemnation codes, was not recorded as "FSIS antemortem condemn".
- The "Primary Reason for Submission" for samples that did not have a condemn code was recorded as "FSIS antemortem condemn".
- The "Primary Reason for Submission" for samples collected from sites other than slaughter, 3D-4D, or rendering facilities, was recorded as "FSIS antemortem condemn".
- The "Primary Reason for Submission" for samples not associated with a foreign animal disease investigation, or not collected by NVSL personnel, was recorded as "Highly suspicious for BSE".
- The "Primary Reason for Submission" for samples that had clinical signs other than "clinically normal" was recorded as "Apparently healthy adult at slaughter".
- The "Primary Reason for Submission" for samples that were recorded to have various clinical signs was recorded as "Dead of unknown cause".
- The "Primary Reason for Submission" for samples not collected

by diagnostic or public health laboratories was recorded as "Rabies suspect".

- The "State of Origin" was missing.

Subsequent to data reconciliation measures, the NSU initiated production of comprehensive weekly and monthly surveillance reports that address surveillance streams, demographic and geographical data. These reports are intended for select APHIS officials. Samples which had any of the following "Primary Reason for Submission" were categorized as targeted regardless of age or other criteria: "highly suspicious for BSE", "CNS signs", "Rabies suspect", or "FSIS antemortem condemn" with a condemn code of "CNS signs" or "Rabies". The remaining samples were only considered targeted if the animals were recorded as 30 months of age or greater, or 24-29 months of age with an erupted second incisor. If derived from animals of appropriate age, samples with a "Primary Reason for Submission" of "Nonambulatory", "Dead", or "Other clinical signs that may be associated with BSE" were categorized as targeted. "FSIS-antemortem condemn" samples were recorded as targeted only if nonambulatory, injuries, tetanus, dead, or moribund was recorded as the condemnation code. Criteria that remove a sample from the targeted population included samples with the results of "Not Detected, Not Obex", "Not Tested", those in which results were missing, and the remaining

samples that did not meet standard for inclusion in the targeted population. Measures portrayed by the weekly reports, in tabular and graphical format, include weekly and cumulative numbers of targeted samples, non-targeted samples, "not detected, not obex" samples, and total samples stratified by collection site or submission type (corresponding to "Primary reason for submission"). Weekly and cumulative percentages of targeted samples by collection site and submission type are presented. Cumulative numbers of targeted samples are listed for each collection site and are stratified by submission type. Finally, cumulative numbers of targeted samples are also listed for each region, and are presented by region of collection site and region in which the animal last resided.

A final report of the enhanced surveillance results will also be produced by the NSU for select APHIS officials. The final analysis is subject to review by OIG, Harvard, and USDA/ORACBA, prior to release. This report is intended to allocate samples from BSE surveillance efforts of the last 7 years into the four surveillance streams provided by the OIE Code,[55] so as to establish the degree with which U.S. surveillance efforts have met requirements for "Type A" surveillance. Additionally, an estimate of BSE prevalence in the U.S. will be calculated using the BSurvE surveillance model.[77]

Strengths:

1. Frequency and timeliness of reporting has been optimal. Delays in reporting have been dictated by database structuring and implementation.

2. The depth of surveillance reports is sufficient. The information provided by reports is succinct, yet plentiful, and is relevant to policy makers. The means of presentation is effective.

Areas for Consideration:

3. Sample data that has been systematically collected, but not used in any analyses, should not be requested in future surveillance programs. Examples of unused parameters include specified breed and neuter status. However, the relevance of such data to objectives outside the surveillance program should be considered before discarding certain parameters.

4. The validity of sample data that required retrospective reconciliation, in some cases long after sample collection, is questionable. Since maintenance surveillance will place more emphasis on the quality of data per sample, consideration should be given to excluding samples with inadequate sample data from testing and/or analysis.

5. Reports do not analyze temporal trends in sample origin. Temporal

analysis may have benefited the recognition of collection site types with participation rates that were stable over time. However, since there is no evidence supporting seasonal variability of BSE incidence, potential temporal bias has little impact on interpretation of surveillance results. In addition, the OIE surveillance standards do not require consistency in surveillance stream yield over time.[55]

Response to a Positive Case

Details: Measures involved in response to a positive case are primarily the responsibility of APHIS, since this agency has been designated by Congress as the lead agency in response to foreign animal diseases. However, given the cross-jurisdictional implications of BSE in US cattle, efforts involve cooperation with the FDA.

The USDA/APHIS BSE Response Plan supplies comprehensive instructions for events to follow the recognition of BSE in US cattle.[78] As previously discussed, consequent to the identification of a presumptive case of BSE, an NVSL pathologist will hand carry the sample to an OIE reference laboratory for confirmation. At this time,

the USDA/APHIS BSE Response Plan is initiated; NVSL is the organization responsible for activating this process. APHIS:VS deputy administrator is informed of a presumptive diagnosis, and of OIE confirmatory test results that follow from 24 to 96 hours later. Working with the FSIS, Office of Public Health and Science (OPHS) deputy administrator, the APHIS:VS deputy administrator alerts the BSE Response Team leaders. The BSE Response Team informs all APHIS regional directors and AVIC's, regional and field FSIS offices, other Federal agencies, key industry/consumer representatives, and foreign embassies, and issues a press release the day the diagnosis is confirmed. The AVIC for the State from which the suspect animal originated assembles the local VS staff to initiate an epidemiologic investigation that traces the progeny and adult herdmates of the suspect animal. The AVIC coordinates with the State Veterinarian to quarantine the suspect animal's herd of origin and progeny.

The FDA BSE Emergency Response Plan Summary describes procedures and delegates responsibilities that are relevant to protect FDA-regulated products or ingredients from the BSE agent.[79] A BSE Emergency is instated in response to a presumptive or confirmed BSE diagnosis in the US, or to any report of a disease caused by the BSE agent in other mammalian species in the US. According to this plan, the BSE Emergency Operations Center (EOC) is notified by APHIS

when a presumptive BSE case is identified. The EOC cooperates with APHIS to determine the origin of the affected animal, and to identify the source of feed and animals potentially contaminated with BSE-agent. The FDA determines if any FDA-regulated products, animal feed, or food for human consumption have been derived from the affected animal or herdmates, and if distribution has occurred. In such an event, an immediate health hazard assessment and action recommendations are produced. In the case of a positive sample collected from a rendering facility, the FDA traces rendered products derived from the animal (if applicable) for disposal and assessment of potential ruminant exposure.

Strengths:

1. Both plans have comprehensively summarized parties that should be notified or involved in response to a BSE case. The sequence of information flow through these parties is satisfactorily detailed by both plans.

2. The temporal succession of actions in response to a BSE case is presented by both plans. Both plans promptly initiate actions.

3. The intended responses to a case of BSE address relevant objectives, mainly to identify and safely dispose of index and related BSE cases in order to prevent spread of BSE in the cattle

population and protect public health. However, details as to how this goal will be met, including important facets of the subsequent epidemiologic investigation, are only superficially mentioned.

Areas for Consideration:

4. Any potential epidemiological investigation into BSE cases is presently limited by the cattle identification systems in place in North America. The specific methods by which this limitation will be addressed should be mentioned by the response plans.

5. Emergency response plans should more clearly address the reasons for action taken such that events are validated by scientific knowledge of the disease and misimpressions by the public can be avoided.

6. Details as to the fate of the affected birth or feed cohorts are not clearly elucidated in either response plan, especially in concerns to extent to which the index herd and herds containing progeny of the affected animal will be depopulated, indemnity fees for producers of these herds, and testing protocol for epidemiologically related animals. These actions should be addressed, and the reasons for these actions made clear, especially if they are in excess of what is recommended by the OIE (i.e. immediate culling of animals as opposed to exclusion from food and feed chains at the end of their

lives). Such transparency will improve acceptability of the surveillance program and will defuse disproportionate fears associated with BSE surveillance.

7. The USDA/APHIS BSE Response Plan was formulated prior to the identification of BSE in U.S. cattle. This plan should be updated to reflect current political sentiments.

Analysis of Qualitative Attributes

Simplicity

From the aspect of disease surveillance, simplicity refers to the structure and ease of operation. Additionally, simplicity denotes the ease with which the system is understood and implemented.

Simplicity of the Enhanced BSE Surveillance Program is high, regardless of its breadth which involves several sample sources and an immense sample size. The structure of the system is uncomplicated, the steps of the surveillance process are distinct, and the responsibilities of parties involved in surveillance are clear. Practices for sample collection are conserved among sample sources, and testing procedure is uniform between diagnostic laboratories. Furthermore, the amount of information necessary to establish diagnosis is minimal.

The following areas of the surveillance program detract from its simplicity and could be improved, as mentioned elsewhere in this document:

1. The definition of the targeted population is ambiguous (refer to "Targeted Population" sections 3-5).
2. Data form trafficking is too complex (refer to "Data Collection Forms" sections 5-6, and Figure 2.3).
3. Several data items collected are beyond the scope of what is needed, or what can be accurately completed (refer to "Data Collection Forms" section 8).

Confusion emerging from poor understanding of the targeted population is evidenced by the fact that not all animals tested counted toward surveillance goals. The number of samples that were tested, but later were excluded from data analysis for reasons other than sample quality ("Not detected, not obex" sample"), amounts to 19,455 (3.5%) as of December, 2005.

Flexibility

Flexibility is the ability of a surveillance system to adapt to change in goals, process and structure, resources, and needs of end-users.

The extent to which the Enhanced BSE Surveillance Program can accommodate modifications is unclear. However, given its simplicity, the system seems capable of responding to new challenges. The program was capable of addressing data reconciliation issues related to major data collection forms and database adjustments during its term.

The following subjects have inherently reduced flexibility:

1. The program requires the involvement of multiple government branches. Although this is necessary, consideration should be given to various exceptions made for FSIS (refer to "Targeted Population" sections 9, 11 and 12, and "Reporting of Test Results" section 4).

2. Aside from diagnostic laboratory practices, there is a fundamental lack of protocol that addresses changes in the surveillance program (refer to "Communication Pathways and Feedback Mechanisms"). The notification process for adjustments in procedure is not standardized. Changes are rarely documented at sample collection sites.

3. Formal communication pathways and feedback mechanisms are sparsely implemented or detailed in the surveillance plan (refer to "Communication Pathways and Feedback Mechanisms").

The program may be perceived as too flexible in regards to providing indemnity for samples that are not derived from animals of the targeted population, or samples for which inadequate sample data were recorded.

Acceptability

Acceptability indicates the willingness of individuals or organizations to participate in the surveillance system. Acceptability is crucial for surveillance programs that rely on voluntary participation. Acceptability is reflected by accurate, consistent, and timely data.

Since willingness to participate is essential for the generation of accurate data, incentives were provided by the Enhanced Surveillance Program. Monetary incentives seem to have enlisted the participation of an adequate number of collection sites in each state, and

testing sites in each region. Most parties involved in surveillance have accepted the program since incentives are generous.

The following topics underscore areas of the Enhanced Surveillance Program that impair its acceptability:

1. The level of fear and apprehension the general public has for the potential identification of BSE is disproportionate to its risk. Resolution of this issue relies on execution of extensive educational programs that detail the public health risk of BSE, the importance of BSE surveillance, and the potential consequences should a case of BSE be identified.

2. The data collection forms impart a significant time burden when properly completed and forwarded (refer to "Data Collection Forms" sections 5 and 8). The poor acceptance of the forms used by the surveillance program has had a negative impact on data quality. The rate of missing data parameters (degree of form completion) provides a crude estimate of data form acceptance. Data forms are estimated to average 2 non-completed parameters per form (refer to "Data Collection Forms" section 13).

3. The program offers no assurance of confidentiality, in regards to involvement in surveillance or test results, to its participants and stakeholders.

4. There is no assurance of consequences associated with response to a positive case that is offered by the program to its participants and stakeholders (refer to "Response to a Positive Case" section 6).

5. A disincentive exists for the support or cooperation of cattle producers (refer to "Sample Collection" section 8).

6. A disincentive exists for the enlistment of slaughter and disposal facilities (refer to "Sample Collection" section 14).

7. Parties that are obligated to participate in surveillance, especially FSIS, have been reluctant in some aspects (refer to "Targeted Population" sections 8, 11 and 12, and "Reporting of Test Results" section 4).

8. Formal mechanisms for the system to address suggestions or comments from its participants have not been provided.

Representativeness

Representativeness is the extent to which the surveillance system accurately addresses the occurrence and distribution of the disease over time. Hence, representativeness concerns the quality of demographic data, and the degree to which targeted population was covered (degree to which the sampled population corresponds to the population for which results will be generalized). Representativeness is essential to defend the validity of surveillance

findings as it provides assurance that only animals representative of the targeted population have been tested and included in data analysis, and that high-risk animals have not been systematically neglected.

The geographical apportioning of minimal sample goals has been an effective mechanism to ensure that there is reasonable coverage of the nation's herds. All regions have complied; goals were nearly met by all regions after 12 months of enhanced surveillance (Fig. 2.6). Therefore, the minimal number of samples collected by region roughly correlates to the known targeted cattle population distribution. However, some regions may have been over-represented, as certain regions excelled allotted goals. Additionally, there was disproportionate representation of the targeted population in all regions (Table 1).

The number of samples categorized as targeted (530,489 as of December, 2005) exceeds the estimated size of the targeted population (445,886, see IV.B), and the number of samples needed to detect one or more BSE cases with a design prevalence of 1 case per 10,000,000 adult cattle and 99% confidence (268,500, see IV.B). Although the accuracy of the targeted population size estimate remains uncertain, it appears that a census-like sampling strategy has been productively employed. This has probably somewhat reduced the effect of non-random sampling strategy since no targeted animals have been purposefully excluded from surveillance.

Since the types of collections sites enlisted by the surveillance program are diverse and ample, there are no recognized targeted subpopulations that may systematically be excluded from surveillance. However, a few major avenues through which targeted animals may escape surveillance have been summarized in Figure 2.7.

The number of animals designated as clinical suspects totaled 2719 as of December, 2005. The expected number of clinical suspects was about 3,095 (0.6% of approximately 515,861 targeted samples, as of December, 2005). This estimate was based on EU data, and is described in detail.[72] Outdated OIE code estimated that 1% of cattle would die per year and 1% of dead cattle (4,500 for the US population) would be clinical suspects. Another means to estimate the number of expected clinical suspects is to use NAHMS's data. The Dairy -'02 NAHMS's estimate on "incidence of deaths in cows due to lack of coordination or severe depression" and the Beef -'99 NAHMS's estimate on "percent of all cattle placed that developed central nervous system problems" were 1.4% and 0.4%, respectively, resulting in more than 4,500 expected clinical suspects.[80, 81] Based on all three approaches to estimate expected numbers of clinical suspects, it seems these animals have been inadequately represented through surveillance.

The representativeness of the Enhanced Surveillance Program is negatively influenced by the following features:

1. Sampling is not random since participation in the surveillance program is voluntary (refer to "Sample Collection" section 7).

2. The exact distribution of cattle in the targeted population is unknown. This precludes formulation of an accurate strategy to optimize representativeness.

3. The population sampled by this system does not evenly represent the geographical distribution of cattle in the U.S., as there is sampling bias favoring animals within the target population that are in closer proximity to certain sample collection sites, especially salvage slaughter facilities, rendering facilities, or diagnostic laboratories (refer to "Sample Collection" section 9). Additionally, there is poor recruitment of samples from on-farm cattle that pertain to the targeted population (refer to "Sample Collection" section 8). A study using geographic information system (GIS) applications is needed to determine if the sample collection sites have been appropriately selected based on geographical position relative to the adult cattle population.
4. Sampling bias is introduced by the exclusion of testing decomposed samples (refer to "Diagnostic Strategy" section 12).

5. Sample data has questionable validity (refer to "Sample Collection" section 13, "Data Collection Forms" sections 4, and 7-11, and "Data Analysis and Dissemination"), therefore, the extent to which the sampled population represents the targeted population is uncertain. Field observations indicate data quality is poor, however, assessing the reliability of form completion would require special studies, such as re-interviews of those involved in form completion, that are outside the scope of this evaluation. However, forms are often submitted without

completion and discrepancies between raw and electronic data have been identified (refer to "Data Collection Forms" section 13)

6. The degree of representation of the targeted population by collection site is confounded by non-uniform geographical outreach to producers and veterinary practitioners, in attempt to encourage program compliance and to raise awareness for the targeted cattle population.

Identification of slaughter or salvage slaughter facilities that could have been involved in surveillance, but weren't, and estimated numbers of targeted animals processed by such facilities, would be beneficial for assessment of acceptability and representativeness of the program. However, these tasks are considered beyond the scope of the present evaluation.

Timeliness

Timeliness reflects the speed between steps in a surveillance system. Timeliness pertains to the interval between disease identification and reporting, evaluation (identification of disease trends, effectiveness of control measures, etc.), and response (implementation of control measures).

Timeliness in a surveillance system for BSE is imperative to maximize the productivity in tracing epidemiologically related animals and by-products. The timeliness of the Enhanced Surveillance System is exceptional from every aspect. Timeliness is

encouraged by the centralized system for disease reporting and data flow that is employed by the surveillance program. Additionally, oversight systems have been established to follow-up on cases for which test results are not promptly recorded.

Sample testing is expeditious (refer to "Reporting of Test Results" section 1 and "Diagnostic Strategy" sections 4-6). Including IHC, the mean days between sample collection date and data entry of test result is 5.6, and the mean days between initial data entry of sample data and entry of test result for samples is 2.5 for samples collected between June 1, 2004 and January 2, 2006. This timeframe is especially impressive considering that diagnostic assays that could be performed on-site are not available for TSE diagnosis.

The time to production of surveillance reports has been excellent, especially considering the short timeframe that was allotted for database design and implementation. Although production of surveillance reports was initially hampered by construction and adjustment of the database, it has since been appropriate, with reports being generated at regular intervals.

The time to respond to the positive case was initially delayed due to deficiency in testing strategy. The animal was first tested in November 2004; although initially "inconclusive" by ELISA, IHC was negative. A confirmatory immunoblot was not pursued until June 10, 2005, due to political reasons. The positive immunoblot was

subsequently confirmed by an OIE reference laboratory on June 24. The epidemiologic investigation of this case was swift with a final report produced in August, 2005.[82]

The following areas impair timeliness of the Enhanced Surveillance System:

1. Form trafficking, corresponding to the progression of data flow, is non-uniform and may prevent timely reporting of test results (refer to "Data Collection Forms" section 6, and "Reporting of Tests Results" sections 4-5).
2. The designated parties for diagnostic laboratories to notify of test results are not standardized. Inclusion of excessive parties may delay result notification to the principal party invested in test results (refer to "Reporting of Test Results" section 4).
3. Bottlenecks that occur with data entry interfere with reporting of test results (refer to "Reporting of Test Results" section 5).

Usefulness

Usefulness indicates the extent to which the surveillance system meets its objectives.

Surveillance data have largely met the objectives of the Enhanced Surveillance Program. BSE has been identified within the native U.S. cattle population, and the U.S. is assured of its BSE status. The single affected cow identified to date was considered subclinical, and has been successfully traced to its herd of origin.[82] USDA and the State of Texas extensively traced cattle at risk, including, the birth cohort, feed cohort, and progeny of the positive cow born within two years prior to the positive test. The majority of animals

of interest were located, and all those still living were euthanized and tested negative for BSE. Therefore, the epidemiologic investigation of the positive animal resulted in events to control disease, however, reports which address investigation into feed source, or which summarize records on rendered products derived from cattle at risk, are not available to date. The prevalence of BSE in the U.S. has been estimated by applying surveillance data to the BSurvE model.[72, 83] Estimating the magnitude of the disease within the U.S. has improved the understanding of epidemiology of the disease problem. The low prevalence seems to support the effectiveness of control measures implemented by the U.S. to prevent BSE. Suveillance data from the period of enhanced surveillance and prior years have been applied to OIE Code,[55] and have indicated that the U.S. practices exceed Type A surveillance standards.[72] Maintenance surveillance has yet to be designed, however, adopted strategies will be dependent on findings of the present system.

Since BSE is exceptionally rare in U.S. cattle, surveillance data could not be used to detect spatial, temporal, or demographic trends in occurrence of disease or risk factors associated with disease.

Summary

Effectiveness of the Surveillance Program

The present surveillance program has been effective in that it has largely met its stated objectives. A low prevalence of BSE in native US cattle has been verified. Results demonstrate that US surveillance practices meet OIE standards. The single animal affected with BSE was traced to its herd of origin. Areas of the surveillance program which are vulnerable to international criticism mostly arise from the dependency of our program on voluntary and cooperative participation and from the deficiency in a reliable animal identification system.

The extent to which the surveillance program meets APHIS/VS surveillance standards[1] has been difficult to determine. The purpose, rationale, and organization of the enhanced surveillance program are described by various fact sheets, reports, and plans; no documents exist which adhere to guidelines provided by VS surveillance standards. Several details could be assembled for the purpose of this evaluation, however, it is recommended that a single written surveillance plan adopt a comprehensive and inclusive format as detailed by VS surveillance standards. Items that have not been addressed by the present surveillance plan and guide include: disease description (etiologic agent, distribution, clinical signs, pathological findings, epidemiology, economic impact, and methods for control), expected outcomes (with emphasis on description of actions taken when a positive animal is identified), stakeholders and responsible parties, data analysis and interpretation, and data presentation and reports. Items that have been addressed, but

require further detail, or need to be more clearly stated, include: purpose and rationale of surveillance, surveillance objectives/goals, case definitions, and description of the population at risk.

Action Items for Consideration

The present evaluation has identified several areas that should be considered while designing a maintenance surveillance program. Since maintenance surveillance will involve a drastic reduction in sample size, resources need to be focused on a higher proportion of valuable samples (samples allotted maximal points by the OIE code). Additionally, measures should be taken to improve acceptability and representativeness of the system, with emphasis on improving sample data validity, increasing the number of clinical suspects captured by surveillance, and minimizing geographical bias. The following items suggest means by which the quality and efficiency of surveillance could be improved:

1. Future surveillance plans should direct goals towards sample points, instead of sample numbers, since points are used to apply surveillance results to international surveillance standards and certain epidemiologic models. As surveillance results are applied to the OIE point code, collecting high sample numbers does not directly correlate to surveillance productivity; sampling for quantity is therefore a considerable waste of resources. Some regions have considerably higher average sample value than other regions (see Table 2). The reasons for this finding remain unclear, but anecdotally, some productive areas

are more dependent on on-farm sample collection or use APHIS employees to record sample data at contracted collection sites. Points allotted per sample are increased by collecting from relatively higher risk animals, and augmenting the quantity and quality of information recorded with each sample. Surveillance streams which produce minimal points per sample should be abandoned, especially if the cost per sample approximates that for point-rich samples.

2. Since sample points are allotted according to surveillance streams described by the OIE code, these streams should be adopted by data collection forms used by a maintenance surveillance program. Data accuracy would be improved and data analysis would be simplified if allocation of samples to the appropriate stream could be done in a prospective fashion, versus retrospectively based on other data parameters. For example, the streams could replace the "Primary Reason for Submission" parameter on data collection forms (refer to "Data Collection Forms" section 9). These streams could also be used to replace or improve clarity of the present ambiguous description of the targeted population in the surveillance plan and guide (refer to "Targeted Population" sections 3 and 5).

3. Efforts should be made to improve collection of clinical signs data so that average sample value is increased and maximal numbers of clinical suspects are identified (refer to "Representativeness"). Efforts could involve selective enlistment of cooperative sample collectors, selective enlistment of collection site types which have historically provided ample data, education of sample collectors on the

importance of such data, rewards for accurate or thorough data recording practices, cautionary statements on data forms regarding consequences of incomplete or falsified data, or audits. A statement which calls attention to the importance of clinical sign data could be added to data collection forms. In addition, education measures supplied to those involved in sample collection could be shifted from sample collection technique and aging cattle by dentition to recognition of possible clinical signs consistent with BSE.

4. Sample collection efforts should be focused towards high-risk animals which have high sample point values. For example, samples should not be collected from animals under 30 months of age, regardless of clinical signs or collection site (refer to "Targeted Population" sections 4, 8 and 9). No apparently healthy animals should be sampled.

5. Maintenance surveillance should attempt to maximize collection of on-farm samples. Such an approach will minimize geographical bias, therefore improving representativeness and the validity of surveillance results, and will be capable of collecting samples from all surveillance streams, especially clinical suspects.

6. Efforts to enlist accredited veterinarians for sample collection should be enhanced in order to improve sample data quality, average point value per sample, uniform geographical representation, and representativeness of the system. Veterinary expertise will aid in identification of clinical suspects and will improve the

accuracy of recorded clinical signs data (refer to "Data Collection Forms section 10). The importance of diligence in monitoring to detect cattle with clinical signs compatible with BSE is emphasized by the efficiency of passive surveillance in the United Kingdom.[24] Since most farms are serviced by a veterinarian, geographical sampling bias will be reduced (refer to "Sample Collection" section 9). Given the perceived integrity associated with the profession, data supplied by veterinarians will be more credible than other parties receiving financial reward for sample contribution (refer to "Sample Collection" section 13). Additionally, veterinarians may serve as sentinels to educate producers and consumers on the importance of a valid surveillance system in protecting U.S. beef.

7. FSIS should continue to be involved in sample collection efforts. Slaughter roughly maintains geographical representation since presumably all farms use slaughter services. Additionally, casualty slaughter receives the second highest point allotment of all surveillance streams, whereas fallen stock, usually collected from disposal-type facilities, receive less points. However, should interest be maintained in collection of fallen stock samples, a significant number of such samples are also presented to slaughter and could be included in surveillance efforts without enlistment of disposal facilities.

8. The data quality of FSIS antemortem condemns that are contracted to be collected off-site needs to be improved (refer to "Sample Collection" section 11). Consideration should be given to requiring that the party that selects an animal for

surveillance complete the Surveillance Data Collection Form, excluding providing a BSE Referral Number or Sample ID.

9. Sample collection efforts should de-emphasize the contributions of rendering, 3D/4D, or other disposal-type facilities. These facilities are not positioned to maintain even geographically representative of the cattle population. Relying on these facilities will introduce spatial bias since there are many regions that are not serviced by such facilities. In addition, the value of samples derived from these sources is minimal since fallen stock samples are of little relative value, and since little animal information, including history and clinical signs, is available when animals are presented dead. Enlistment of these facilities will not be needed if FSIS remains involved since slaughter will also recognize a significant number of fallen stock. As disposal-type facilities are largely restricted to collecting samples from fallen stock, disease is unlikely to be recognized in samples derived from these facilities. Although the BSE cases identified in Canada were clustered around a rendering facility, there is no scientific evidence to suggest that geographical positioning of cattle relative to rendering facilities is associated with increased risk for BSE. Furthermore, although rendered products of BSE-positive animals may present an opportunity for disease spread, the appropriate control measures are in place (refer to "Regulatory Actions for BSE in the U.S.") such that monitoring animals submitted for rendering for BSE is not an efficient means to control disease.

10. Attempts should be made to increase the proportion of samples collected by APHIS personnel. Since APHIS employees will be more accountable for accurate selection of targeted animals and form completion, data quality and accountability will be improved. Since government employees will not be financially rewarded for each sample, there are no incentives to falsify data (refer to "Sample Collection" section 13). Also, APHIS employees can be trained and monitored more effectively, resulting in improved data validity. Otherwise, it will be difficult to convince trading partners to accept the validity of the surveillance program should it become apparent that inadequate or inappropriate cattle possibly constituted a portion of the testing program.

11. Facilities where those collecting samples cannot complete data forms should be excluded from future surveillance, or should be allotted PC tablets so that form completion can be done at the time of sample collection (refer to "Data Collection Forms" section 7). Examples of such facilities include those employing non-English speaking personnel for sample collection, or those with working conditions that preclude form completion in the sample collection area (certain rendering and salvage slaughter facilites). These circumstances preclude accurate sample data collection.

12. The stigma that presently encumbers producer participation needs to be reduced so as to improve acceptability of the system and to increase the number of clinical suspects captured by surveillance (refer to "Sample Collection" section 8).

Assurance of predefined science-based consequences for the detection of a positive case should be provided to alleviate the negative incentive for participation.

13. The quality of data would be improved by enhancing the clarity of submission forms and increasing the efforts directed at training or supervising those completing the forms.

14. The workload associated with data collection form completion needs to be reduced in order to encourage accurate and detailed data recording practices. Additionally, removal of excessive parameters will call attention to the clinical signs section. Forms could be simplified by removing parameters that have served little use in data analysis or animal tracking. The "Primary Reason for Submission" section should be improved or replaced (refer to "Data Collection Forms" section 9). Unwarranted animal source and identification requests should be eliminated (refer to "Data Collection Forms" section 8).

15. Consideration should be given to reducing the size of the FSIS Condemnation Code section of the data collection form. Completion of this section may detract from completion of the clinical signs section, which provides much more valuable data (refer to "Data Collection Forms" section 14).

16. A formal feedback mechanism which recognizes incomplete forms, incorrectly completed forms, and redundantly completed forms, needs to be established.

17. The trafficking of submission forms and results could be simplified or at least standardized across regions (refer to "Data Collection Forms" sections 5-6, "Reporting of Test Results" section 4, and Figure 2.3). Diagnostic laboratories should not be required to forward submission forms under any circumstances. When forms are completed electronically, consideration should be given to the importance of forwarding printed copies to AVIC offices since the data is available through the NAHLN website. When forms are completed by hand, hard copies should be filed at the site of data entry. Consideration should be given to minimizing the number of parties diagnostic laboratories should be responsible for informing of test results.

18. Financial incentives need to be essentially equivalent between geographical areas and collection sites of a given type to reduce sampling bias.

19. To reduce geographical biases in sample data patterns, uniform outreach between states is needed and is best accomplished using national oversight. Relevant outreach materials include those addressing training for sample collection, or those which encourage enlistment of various parties potentially involved in sample collection.

20. Case definitions need to be more clearly stated in future surveillance plans (refer to "Case Definition"). These should address how to interpret discrepant test results. In addition, the diagnostic power of histology should be mentioned.

21. An official feedback mechanism which corrects poor sampling practices (i.e. submission of decomposed or inappropriate tissue) needs to be integrated into the system's structure (refer to "Communication Pathways and Feedback Mechanisms" section 4). Such mechanism should not rely on the discretion of parties that might have recognized the deficient sample. For example, in response to a "not tested" or "not detected, not obex" sample, an automated electronic notification directed to the sample submitter could be generated. This notification could describe why the sample was not tested in a standardized format, and would not require extraneous effort from the diagnostic laboratories or the AVIC offices.

22. The selection of test results available at data entry needs to be expanded such that certain results have more meaning. For example, "not tested" samples should also include a reason for not being tested. "Not detected, not obex" results should include a reason why the sample could not be recognized as obex (refer to "Reporting of Test Results" section 3). Additionally, the results should be entered such that data clearly reflect whether a sample was "inconclusive" based off IHC or ELISA.

23. Rules need to be in place so that "inconclusive" and "initial reactor" samples are permanently recorded in the database (refer to "Diagnostic Strategy" section 19).

24. Collection sites should be encouraged to submit samples of any quality, especially from animals that have known clinical histories. The determination of sample testability should always be left to the expertise of diagnostic laboratory technicians (refer to "Diagnostic Strategy" section 13).

Appendix

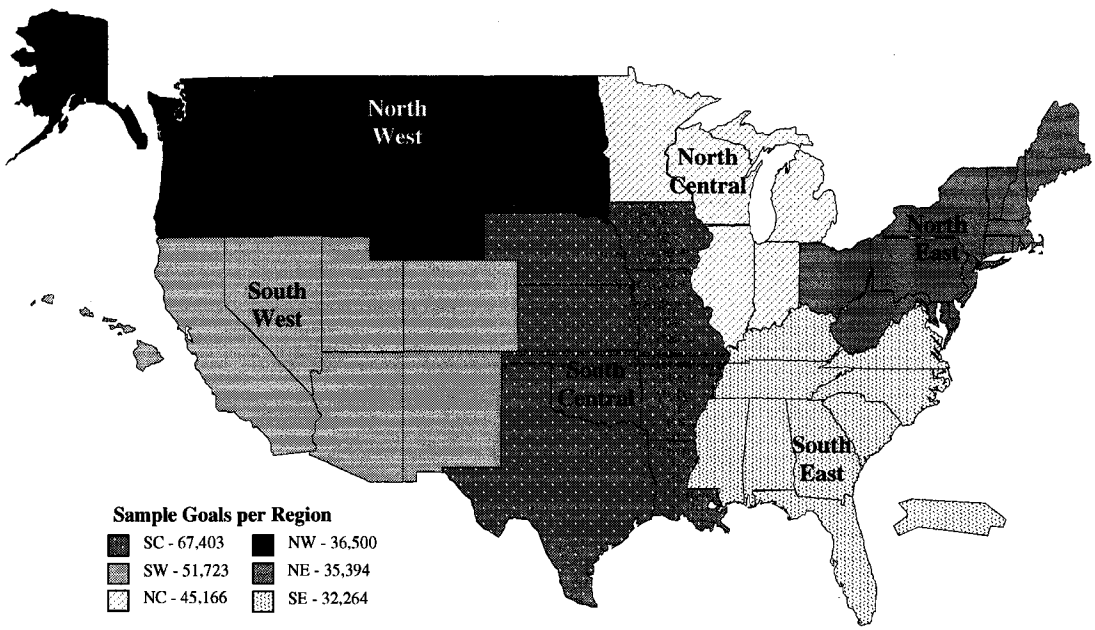

Figure 2.1: Minimal sample allocations by region.

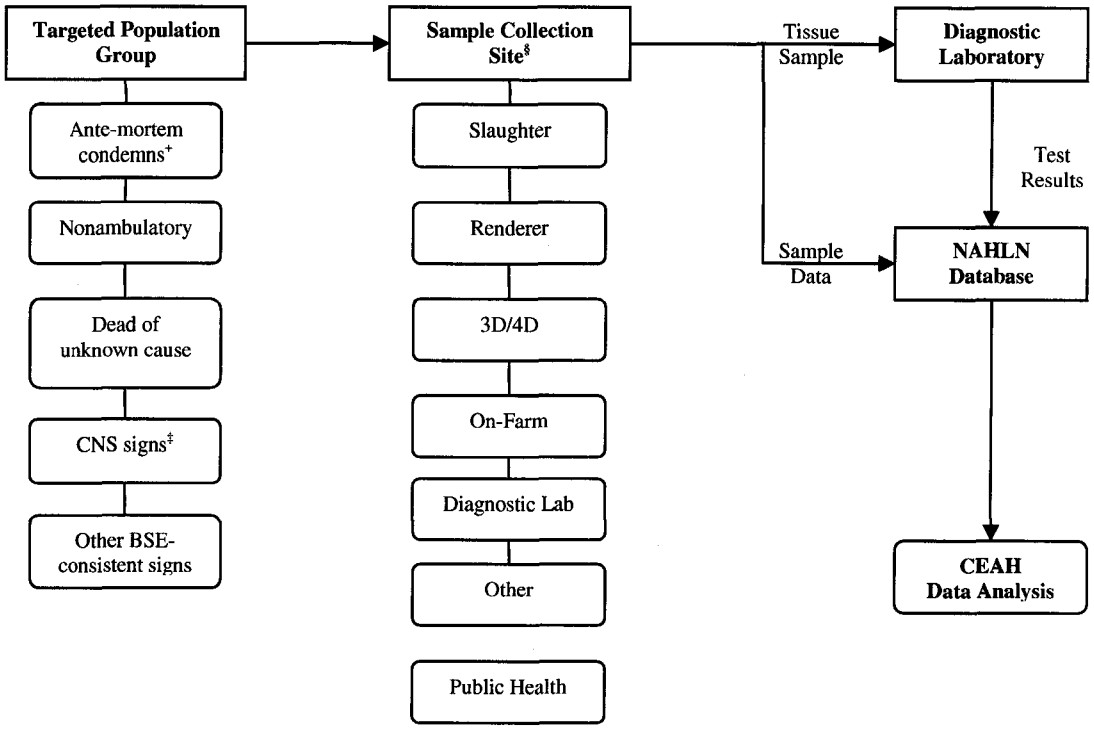

Figure 2.2: General surveillance steps.
§Samples collected by federal or state employee, accredited veterinarian, or contracted, diagnostic lab, or plant employee
+Animals of any age, except veal calves
‡Animals of any age

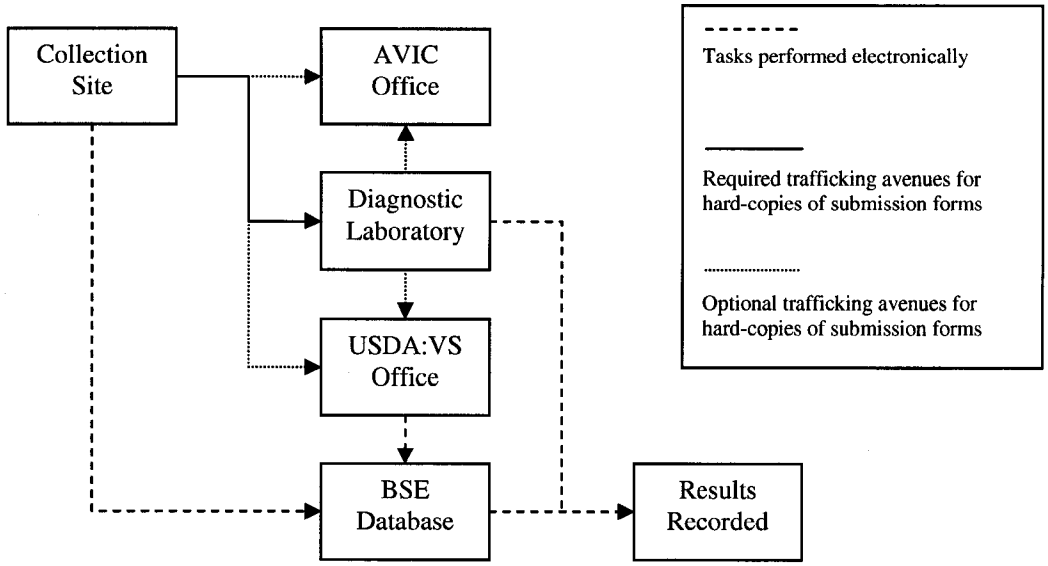

Figure 2.3: Trafficking of Submission Forms. Data from submission forms is entered into the database either directly through electronic submission by the collection site, or indirectly through submission of a hard-copy to a USDA:VS office. Sample data entry must be completed for results to be entered by the diagnostic lab. The diagnostic laboratory must receive sample information to appropriately identify samples; this requires a hard-copy. Some states demand the collection site or diagnostic laboratory forward submission forms to the AVIC office.

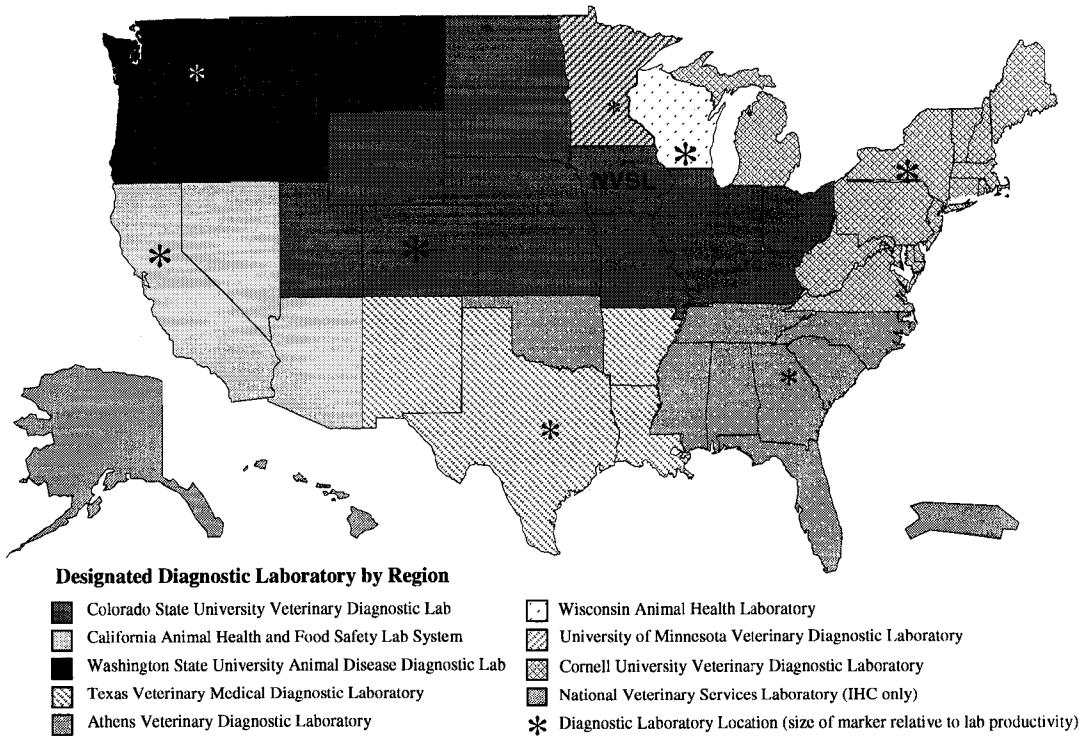

Figure 2.4: Designated diagnostic laboratory by region.

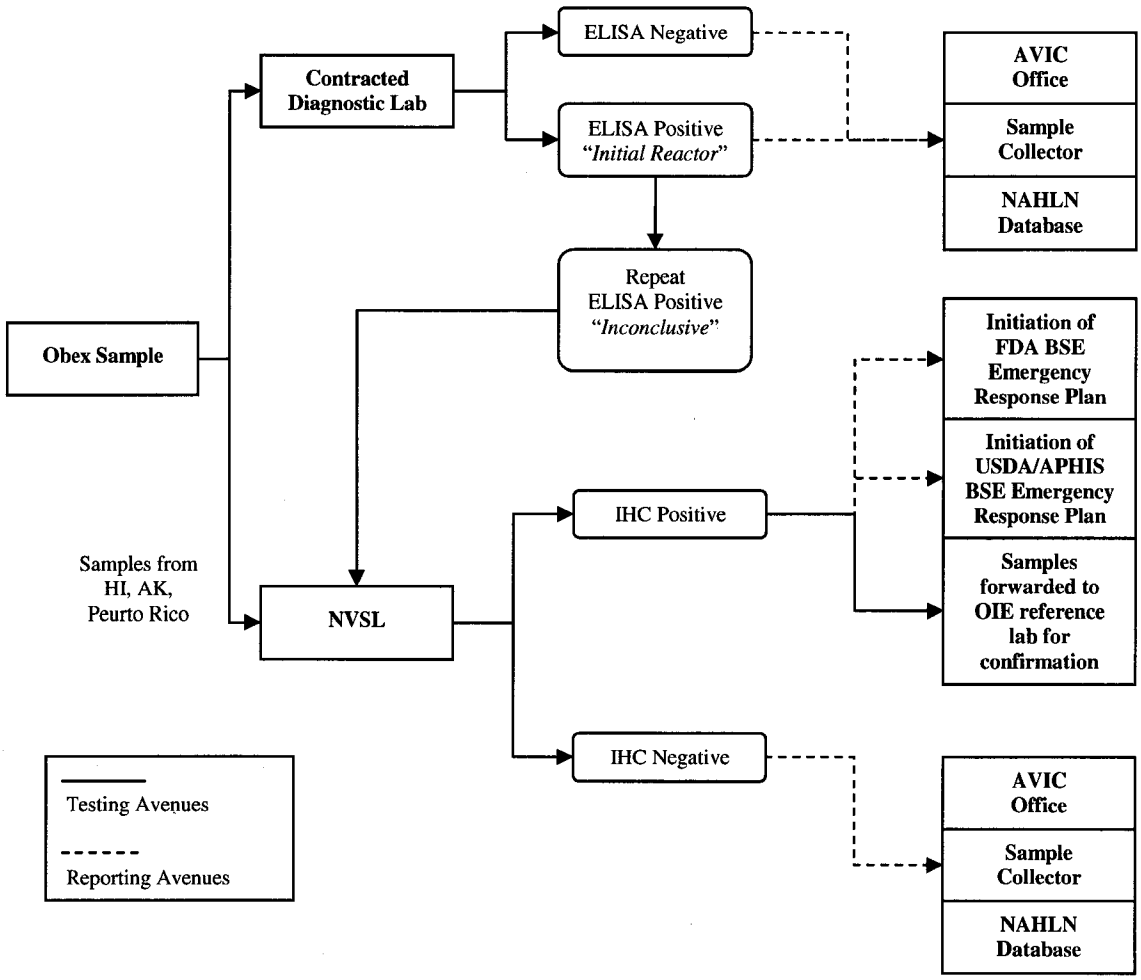

Figure 2.5: Flow chart for diagnostic testing and reporting strategy.

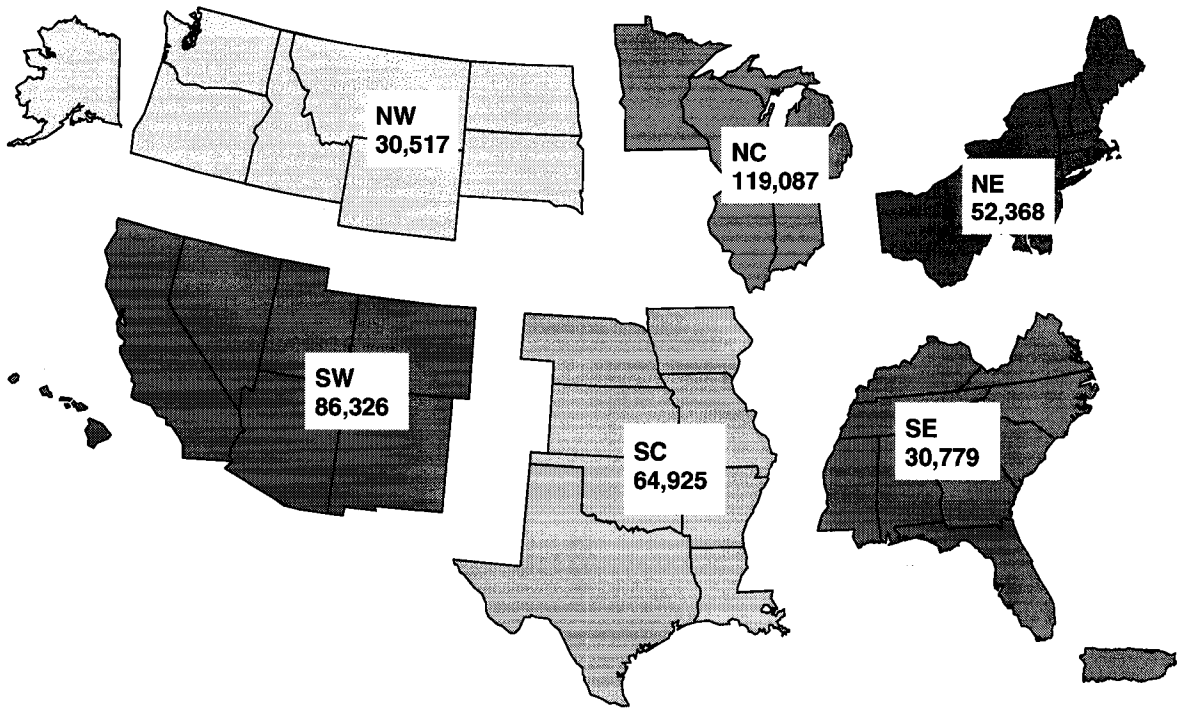

Figure 2.6: Cumulative number of targeted samples tested from June 1, 2004 through June 30, 2005 by region where cattle last resided; adopted from *Preliminary Analysis of BSE Enhanced Surveillance in the U.S. 2004-2005*.[83]

Region	Percent of samples derived from region	Percent of targeted cattle residing in region
NC	30%	17%
NE	14	13
NW	8	14
SC	17	25
SE	8	12
SW	23	19
Total	**100**	**101**

Table 2.1: Sample contributions by geographical region.

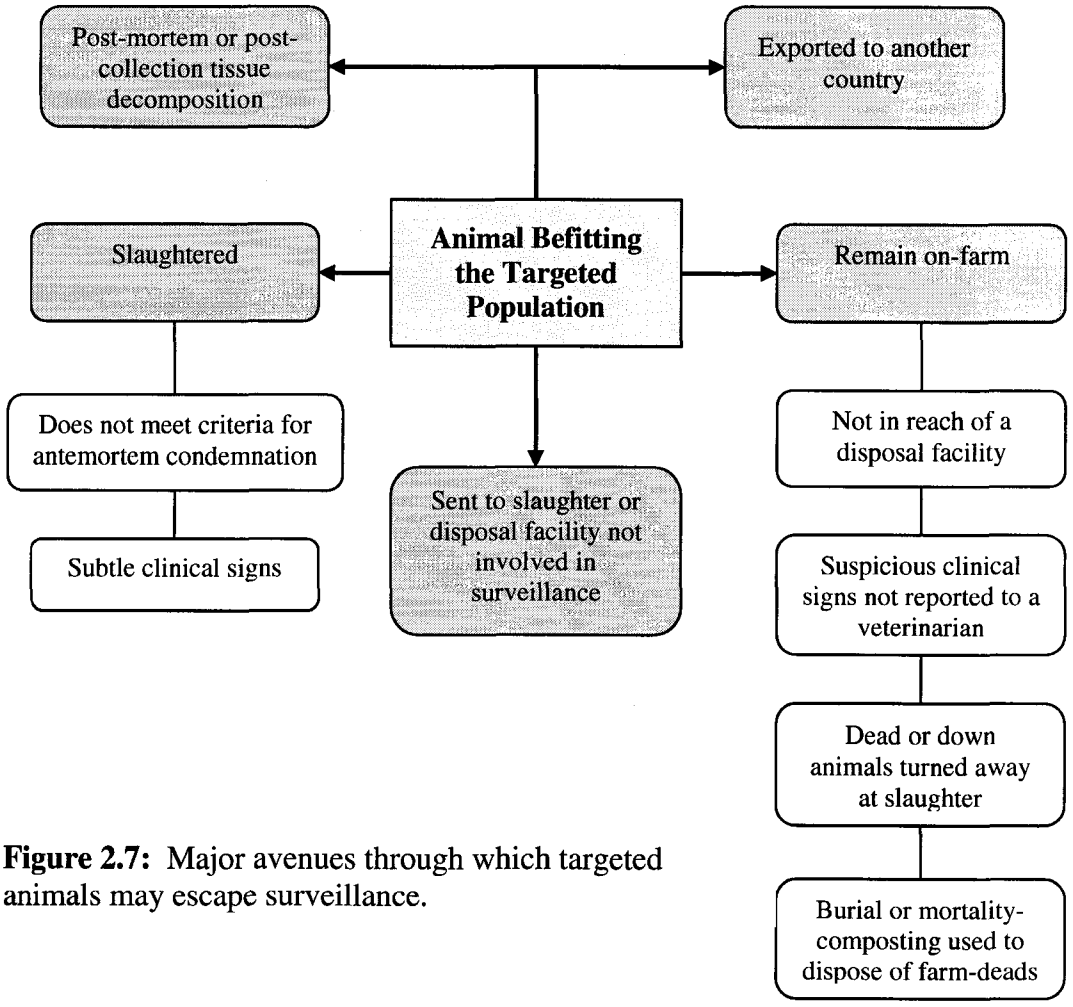

Figure 2.7: Major avenues through which targeted animals may escape surveillance.

Region of Origin	Number of Samples Collected by Surveillance Stream					Total Points	Average Sample Value (Points per Sample)
	Clinical Suspects	Casualty Slaughter	Fallen Stock	Healthy	Total		
NC	147	16,339	138,484	13,576	168,546	403,793	2.4
NE	327	6,101	68,850	17	75,295	544,807	7.2
NW	108	985	41,559	27	42,679	166,875	3.9
SC	405	5,502	85,102	6,061	97,070	452,939	4.7
SE	835	4,223	38,211	22	43,291	939,214	21.7
SW	281	2,213	125,293	1,311	129,098	516,841	4.0
Total	**2,103**	**35,363**	**497,499**	**21,014**	**555,979**	**3,024,468**	**5.4**

Table 2.2. Number of samples and surveillance points obtained by region of animal origin. Note that regions which collected comparatively lesser samples (NE and SE) have accumulated more points relative to other regions. Areas which have collected large sample numbers, but relatively less surveillance points have an excessive proportion of samples derived from fallen stock, and deficient proportion of samples derived from clinical suspects. Since sample costs between regions are roughly comparable, regions with low point yields and high sample numbers have expended considerable resources with little surveillance productivity.

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CHAPTER 3

Bovine Spongiform Encephalopathy (BSE) Maintenance Surveillance Plan

Introduction

The U.S. Department of Agriculture (USDA) has taken aggressive measures to prevent the introduction and potential spread of bovine spongiform encephalopathy (BSE), including conducting surveillance across the United States. Surveillance was expanded in scope and intensity following the confirmation of BSE in an imported cow in December 2003. This expanded surveillance effort was designed to assist in determining the efficacy of existing risk management policies for both animal and public health and more accurately determine the level of disease present in the U.S.

The present plan is intended to inform and educate USDA's partners and stakeholders on approaches to be employed in maintenance BSE surveillance. The plan, while resulting in the reduction of surveillance intensity for BSE in the United States, maintains surveillance at levels that exceed international standards, emphasizes sample collection

from cattle most likely to be infected with BSE if present in the U.S., and retains sample collections from all important surveillance sources.

The plan follows surveillance system design standards and guidelines established by the USDA-APHIS-Veterinary Services, National Surveillance Unit. These guidelines are intended to assist planners and managers in considering specific objectives, design strategies, reporting systems, implementation methods, and long-term system maintenance. The guidelines ensure that the objectives of the surveillance system are predefined, and that the collection, organization, and analysis of appropriate data are considered before implementation. Further, the guidelines allow for review and evaluation to assure that the surveillance is providing the appropriate type and quality of information.

Disease description

Bovine spongiform encephalopathy, commonly known as "Mad Cow Disease", is a transmissible neurodegenerative disease of adult cattle that emerged in Great Britain in 1985,[1] and has subsequently been identified in cattle of most European countries, Canada, the U.S., and Japan. BSE belongs to the group of transmissible spongiform

encephalopathies (TSEs), together with scrapie of sheep, chronic wasting disease (CWD) of free-ranging and captive deer and elk, and Creutzfeldt-Jakob disease (CJD) of humans. TSEs have long latency periods, are untreatable, and currently cannot be prevented by vaccination since there is absence of a host immune response to infection.

Most scientific evidence suggests that prions are the causative agent of TSEs,[2] however, the nature of the prion remains undetermined. A unique characteristic of the prion is its resistance to inactivation by most conventional physical or chemical decontamination methods.[3] The prion consists mostly of protein, largely comprised by a proteinase-resistant, disease-associated isoform (PrP^{res}) of host-encoded prion protein (PrP^{C}).[2,4] The pathogenesis of the TSEs requires the formation of PrP^{res} from PrP^{C}.[5] Interaction with PrP^{res} leads to post-translational conformational modification of PrP^{C},[6-8] resulting in its conversion to PrP^{res}.[9,10] The subsequent pathological accumulation of PrP^{res} in certain tissues defines the TSEs.[11,12]

The pathogenesis of BSE appears to involve a much more restricted tissue distribution of PrP^{res} accumulation than other animal TSEs, having reduced involvement of the lymphoreticular system. Following oral challenge with BSE agent, cattle accumulate

infectivity in CNS tissue, dorsal root ganglia, trigeminal ganglia, aboral ileum, tonsil and bone marrow.[13,14] These tissues, in addition to spleen, thymus, eyes, skull, vertebral column, and mesentery, are designated as specified risk material (SRM) in cattle > 30 months of age, and are considered to represent the greatest risk of BSE exposure to humans and animals.

The origin of the BSE agent is unresolved. Theories have considered derivation from a TSE agent of another mammalian species, such as scrapie,[15] or spontaneous genetic mutation of the bovid prion protein gene.[16] The emergence of BSE coincided with reduced use of hydrocarbon solvents in the production of meat-and-bone meal (MBM) through carcass rendering.[15]

Transmission of BSE is thought to primarily occur through ingestion of feedstuff, especially ruminant-derived MBM, contaminated with the BSE agent.[17,18] Dairy breeds are at higher risk for BSE compared to beef breeds because high concentrate feed rations are more frequently used by dairy operations. Calves born to infected cows have increased risk to develop BSE,[19] especially if born around the time of disease onset in the dam.[20] However, the risk is probably influenced by conserved management practices

where both the dam and calf have been fed concentrated feedstuff containing MBM early in life.[21] Horizontal transmission of BSE between cattle is not believed to occur.

Most cattle become infected within the first 6 months of life.[21] The mean incubation period for BSE is around 60 months, with clinical onset of disease occurring on average at 4-5 years of age.[22] The age range of affected animals is very wide, although BSE is rarely confirmed in animals less than 30 months of age.[23,24]

BSE is invariably fatal. Clinical signs have an insidious onset and are largely nonspecific. Signs that may be associated with BSE include apprehension, ataxia, emaciation, hypersensitivity to touch or sound, head shyness, panic-stricken response, kicking in the milking parlor, reluctance to enter the milking parlor, abnormal ear movement or carriage, increased alertness behavior, reduced milk yield, bruxism, and change in temperament.[18,25] The duration of clinical signs averages 1 to 2 months prior to death or slaughter, but may range from weeks to a year.[18]

Although live animal tests are under development, at present, none are available to reliably detect BSE. Diagnosis is achieved postmortem through examination of central

nervous system (CNS) tissue, and is contingent on identification of characteristic histopathologic lesions, detection of PrP^{res}, or electron microscopic visualization of scrapie-associated fibrils.[26] Because PrP^{res} is the only currently known disease-specific macromolecule, all commercially available diagnostic assays rely on its immunological detection.[27] These assays have limited diagnostic sensitivity in that PrP^{res} accumulation may not be detectable until late during the incubation period, within months prior to onset of clinical disease.[28] Infected animals that are early in the incubation period can only be identified through demonstration of tissue infectivity using bioassay. The only lesions associated with BSE are found microscopically within CNS tissue.[1,29] Lesions develop late in the disease process, roughly coinciding with the onset of clinical signs. These consist of non-inflammatory vacuolar degeneration, or spongiform change, of grey matter and neuronal cell bodies. Astrocytosis and cerebral amyloidosis, features of other TSEs, are unusual with BSE.

Breed-dependent differential susceptibility or incubation period has not been observed with BSE,[30] and there is little variability in the bovine PrP gene.[31] The consistent neuroanatomical lesion profile in the brains of cattle affected with BSE,[29,32] and uniform glycoform ratios of PrP^{res} as determined by immunoblotting,[33,34] suggest the existence of

a single strain of the BSE agent. However, a recently described atypical form of BSE, termed bovine amyloidotic spongiform encephalopathy (BASE), has modified glycoform patterns similar to sporadic CJD in humans, and may represent an alternative strain of BSE agent.[35]

Although TSEs are usually confined to an individual species, concern has arisen for the potential of inter-species transmission of BSE. The BSE agent is widely recognized as the cause of variant CJD in humans, based on epidemiological and mouse inoculation studies,[36,37] and biochemical PrP^{res} characteristics.[33] In addition, natural exposure to BSE agent has led to similar encephalopathic disease in captive wild ungulates and cats,[38] and in domestic cats.[39]

Measures established by the U.S. to prevent new cases of BSE have included a ban on importation of ruminants and at-risk ruminant-derived products from BSE-endemic countries (9CFR94.18), and prohibition of feeding certain mammalian-derived proteins to ruminants (21CFR589.2000). Mitigating measures have been considered to effectively reduce the likelihood of BSE introduction and amplification in U.S. cattle.[40] Food-safety precautions implemented by the U.S. to protect the consumer have included exclusion of

non-ambulatory cattle from slaughter for human consumption (Docket No. 03-025IF), removal of specified risk material from meat for human consumption (Docket No. 03-025IF), and prohibition of the use of injection stunning devices to immobilize cattle during slaughter (Docket No. 01-033IF).

Purpose, rationale, and objectives of surveillance

Animal and public health concerns have led to the establishment of active surveillance programs among other regulatory measures to prevent and monitor disease. Active surveillance for BSE was initiated in the U.S. in 1990. In response to identification of a BSE-affected imported dairy cow in December, 2003, the U.S. Enhanced BSE Surveillance Program was implemented in June, 2004. Through these efforts, a single positive domestic cow, a 12 year-old Brahma cross that originated in Texas and found dead following transport to slaughter, was identified.[10]

Based on data collected in the U.S. over the last seven years, including over a half million samples from the Enhanced Surveillance program, the USDA has developed a preliminary estimate of probable prevalence of BSE among U.S. cattle that was extremely low, projected at less that 1 case per million animals in the standing adult cattle

population, at the 95% confidence level.[41] In addition, the USDA demonstrated that surveillance efforts to date far exceed the World Organization for Animal Health (Office Internationales des Epizooties [OIE]) "type A" surveillance requirements. Prevalence is expected to continue to decline as long as mitigation efforts that maintain low risk for introduction and spread of the BSE agent among U.S. cattle are equivalent to or better than those evaluated by the Harvard Risk Assessment.[40] Because the results of Enhanced Surveillance indicate that BSE is at an extremely low level in U.S. cattle, and because mitigation measures are firmly in place, it is appropriate to end the Enhanced Surveillance efforts, while maintaining a robust system for monitoring the BSE status of the U.S. cattle population. The present plan details the objectives and methods considered pertinent and necessary for maintenance BSE surveillance, should Enhanced Surveillance culminate. In order to maintain confidence that BSE is exceedingly uncommon among U.S. cattle, sampling methods of maintenance surveillance are designed to detect disease should the prevalence rise above 1 case per 1,000,000 adult cattle.

The principal purposes of maintenance BSE surveillance are:

1. To continue to assess and monitor change in the BSE status of U.S. cattle.

2. To provide the mechanisms for early detection of BSE among U.S. cattle.

Expected outcomes

The results of maintenance BSE surveillance will be used for decision-making and policy development regarding design and implementation of future BSE surveillance programs and control efforts. Results will also be used to facilitate contingency plans for national BSE control and response programs and to evaluate the effectiveness of mitigations and control measures which have been implemented to reduce the risk of introduction and spread of BSE among U.S. cattle. The manner in which surveillance data will be used to direct efforts in response to a positive case are described elsewhere.[42,43] Monthly and annual summary reports which describe surveillance findings will be generated.

Additionally, BSE surveillance results will provide reassurance of consumers and international trading partners of the BSE status of U.S. cattle. The USDA has designed this surveillance program to meet or exceed the internationally accepted surveillance practices recommended by the OIE. Compliance of a country's surveillance system with the guidelines provided by the OIE is necessary to assure trading partners that conclusions based on surveillance data are valid.[44] By demonstrating this, the USDA

expects that this robust surveillance program will continue to provide the foundation for market confidence in the safety of U.S. cattle.

Stakeholders and responsible parties

Users of surveillance system information include policy makers (Secretary of Agriculture, Animal and Plant Health Inspection Service (APHIS) administrator, Veterinary Services (VS) deputy administrator), trading partners, Foreign Agriculture Services (FAS), consumer groups, the VS TSE program manager, VS TSE epidemiologists, the National Surveillance Unit (NSU), VS Area Offices, State Animal Health Offices, and data providers (sample collectors and veterinary diagnostic laboratories). In addition to information users, beneficiaries of the surveillance information include the public, the U.S. cattle industry, and industries engaged in export markets for cattle-derived products.

Parties responsible for data application design, development, and implementation include Center for Animal Disease Information and Analysis (CADIA), BSE database Change Control Board, the NSU, VS TSE program manager, VS TSE epidemiologists, and VS

regional directors. Data application support and maintenance will be managed by CADIA, Change Control Board, and regional IT specialists.

Data collection will be performed by a variety of parties. Laboratory data will be derived from the National Veterinary Services Laboratories (NVSL) and laboratories contracted for BSE testing. American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited veterinary diagnostic laboratories participating in the National Animal Health Laboratory Network (NAHLN) may be contracted by NVSL to perform BSE screening tests, provided that their services are needed by the NAHLN and that certain quality assurance standards have been met (as described by the most current version of NVSL protocols GPPISOP3501 and 3303). Sample data will be derived from accredited veterinarians, Food Safety Inspection Service (FSIS) inspectors, veterinary diagnostic laboratory or public health laboratory personnel, and qualified VS personnel (including animal health technicians and those involved with data entry). VS Area Offices will direct efforts for resolution of problematic data.

Cases will be detected through cooperation of NVSL and contracted veterinary diagnostic laboratories. Cases will be confirmed by NVSL and an OIE reference laboratory.

Several parties will be responsible for reporting positive cases along a variety of avenues described elsewhere.[42,43]

Training of sample collectors will be completed by Area Veterinarian in Charge (AVIC) office personnel with oversight by VS regional offices. Training of data entry will be completed by these parties as well as the BSE Help Desk.[i]

Assessment of data quality, data analysis, and interpretation will be completed by the NSU, VS TSE program manager, and VS TSE epidemiologists. Reporting and dissemination of surveillance results will be primarily the responsibility of the NSU.

In addition to standard AMS audit procedures, the NSU will conduct a review of the surveillance system's effectiveness. NVSL will be responsible for quality assurance of laboratory results.

[i]Veterinary Services BSE Help Desk: (866) 370-6611, bse.help@aphis.usda.gov

Population description and sampling methods

Introduction

The following sections detail the methods for a robust and comprehensive maintenance BSE surveillance system that will be adopted at the termination of the Enhanced Surveillance program. The methods have been designed to continue to assess and monitor for change in the BSE status of U.S. cattle, maintain confidence that BSE prevalence is below 1 case per 1,000,000 adult cattle, and provide the basis for developing future surveillance, control, and response efforts, while complying with international BSE surveillance standards. Proposed surveillance methods minimize geographical bias, improve sample data validity, maximize the amount of information collected with each sample, and capture a larger proportion of samples from subpopulations likely to have a higher prevalence of BSE.

The population targeted by Maintenance Surveillance consists of subpopulations of "high risk" cattle targeted by Enhanced Surveillance that offer the highest probability for BSE detection. These consist of: 1) cattle of any age with CNS signs, and 2) cattle ≥ 30 months of age that are condemned during antemortem inspection at slaughter, or are excluded from slaughter due to poor health status (unhealthy or dead due to illness or

injury). In contrast to Enhanced Surveillance, efforts for collecting clinical history data will be improved, and samples from cattle with known clinical history will be preferred over those without. Only a small portion of cattle that are dead and have unknown clinical history will be included in the surveillance sampling strategy; these will only be accepted from FSIS-inspected slaughter facilities and rendering or 3D/4D facilities. During data analysis, samples meeting the target criteria will be assigned to OIE surveillance streams based on clinical signs, sample source, and condemnation code data. The collection site types enlisted by Maintenance Surveillance have been selected based on data collected during Enhanced Surveillance (i.e. average point value per sample, total sample numbers, data validity) and accessibility to targeted animals regardless of collection site location. Enlisted collection sites include: on-farm, veterinary diagnostic laboratories, public health laboratories, FSIS-inspected slaughter facilities, veterinary clinics, livestock markets, and rendering or 3D/4D facilities. All targeted animals that are presented to collection sites will be sampled, except for those presenting to rendering and 3D/4D facilities where a quota of 5,000 samples has been established. Cattle that are identified with the surveillance criteria of "clinical suspect" will be sampled throughout the surveillance period regardless of the avenue through which they present to surveillance, and regardless of the degree with which sampling goals have been met.

Sampling goals have been projected at 40,000 samples, 42,857 OIE points, and 414,285 analytical points per year. As portrayed below, findings from Enhanced Surveillance indicate that the number of targeted cattle available to enlisted collection sites suffice to meet or surpass these goals. However, international standards, suggest that surveillance points be included in the analysis of a country's BSE status for 7 consecutive years. Therefore, for the next 6 years, the points accumulated during Enhanced Surveillance provide considerable elasticity for adaptation to the methods required by Maintenance Surveillance.

OIE Article 3.8.4 provides for the assignment of point values to samples based on the likelihood of testing positive for BSE according to age and surveillance stream (see Table 3.1). Compiled points are used as evidence of adequate surveillance to determine a country's BSE risk level. Note that samples from "clinical suspect" cattle are the most valuable, and it follows that OIE guidelines suggest that the number of clinical suspect samples captured annually should approximate the number collected during a year of Enhanced Surveillance. Conversely, "fallen stock" (comprised largely of cattle that are dead with unknown clinical history) and "routine slaughter" are of comparatively negligible value.

Table 3.1. OIE point values for each surveillance stream by cattle age

	Clinical suspect	Casualty slaughter	Fallen stock	Routine slaughter (Apparently Healthy)
Age ≥ 1 year and < 2 years	N/A	0.4	0.2	0.01
Age ≥ 2 years and < 4 years	260	0.4	0.2	0.1
Age ≥ 4 years and < 7 years	750	1.6	0.9	0.2
Age ≥ 7 years and < 9 years	220	0.7	0.4	0.1
Age ≥ 9 years	45	0.2	0.1	0.0

Sample data from Enhanced Surveillance have been assigned into the four surveillance streams described by the OIE code based on the recorded reasons for submission and clinical signs (see Table 3.2). The majority of points were derived from "clinical suspects", although these samples comprised a small portion of samples evaluated by surveillance (0.4% of samples, 70.3% of points). On the contrary, samples from "fallen stock" comprised the majority of samples, but minimally contributed to overall surveillance points (82.8% of samples, 23.5% of points). Samples collected from slaughter plants, veterinary diagnostic or public health laboratories or on-farm yielded a

high percentage of samples from subpopulations presenting with clinical history compatible with BSE, whereas renderers and 3D/4D facilities yielded a very low percent of such samples. Furthermore, when the average point value per sample was determined by collection site type, it is apparent that on-farm samples, slaughter plants, and diagnostic or public health laboratories consistently produce the most points per sample, whereas samples derived from renderers and 3D/4D facilities are of much lesser value to surveillance.

In summary, collection of samples from cattle with CNS signs or cattle unfit for slaughter (belonging to the "clinical suspect" and "casualty slaughter" surveillance streams), and cattle presenting to collection site types that have most efficiently captured these streams, will be accentuated during Maintenance Surveillance. Conversely collection of samples from cattle that died of unknown cause with no other clinical history (belonging to the "fallen stock" surveillance stream), or from cattle presenting to 3D/4D or rendering plants, will be de-emphasized during Maintenance Surveillance. These proposed surveillance methods will efficiently accumulate surveillance points. Data from 12 months of Enhanced Surveillance indicate that it will be feasible to collect adequate samples numbers from the proposed targeted population by focusing collection efforts on

samples from FSIS-inspected slaughter plants, on-farm, and diagnostic or public health laboratories. Furthermore, these collection site types will provide samples from all geographic sections of the country.

Table 3.2: Average OIE point value per sample, and number (and percent within stream) of samples obtained during Enhanced Surveillance (June 1, 2004 through Nov. 30, 2005) stratified by collection site type and surveillance stream

Collection Site Type	Average Point Value per Sample	Number of Samples (and percent) by Surveillance Stream									
		Clinical suspect		Casualty slaughter		Fallen stock		Routine slaughter		Total	
Renderer	0.9	117	5%	20,542	28%	266,942	58%	2	0%	287,603	52%
3D-4D	1.2	170	7%	29,049	40%	158,729	34%	30	0%	187,978	34%
Slaughter Plant	4.1	378	17%	13,444	19%	1,291	0%	20,977	100%	36,090	6%
On Farm	18.6	1,078	47%	3,758	5%	22,896	5%	2	0%	27,734	5%
Other	2.5	57	2%	5,111	7%	8,683	2%	1	0%	13,852	2%
Diagnostic Lab	57.9	344	15%	390	1%	1,840	0%	2	0%	2,576	0%
Public Health Lab	366.8	146	6%	0	0%	0	0%	0	0%	146	0%
Total Samples		2,290 (0.4%)		72,294 (13.0%)		460,381 (82.8%)		21,014 (3.8%)		555,979	
Total OIE Sample Points		978,145 (70.2%)		83,898 (6.0%)		326,605 (23.5%)		3,619 (0.3%)		1,392,267	
Average Point Value per Sample		427		1.2		0.7		0.2		2.5	

Population description and characteristics

Individual cattle will be sampled for inclusion in BSE surveillance. Inferences derived from surveillance findings will be generalized to the adult U.S. cattle population (i.e. target or inference population), which consists of approximately 42 million adult cattle.[45]

Study (Targeted) Population

Maintenance Surveillance will target subpopulations of the Enhanced Surveillance "high risk" population, which have the highest probability of BSE detection and therefore provide the greatest amount of surveillance information. Because BSE is exceedingly uncommon among U.S. cattle, the chosen targeted population will create intentional bias in the sample frame which favors detection of disease. In contrast to Enhanced Surveillance, cattle that are dead with unknown clinical history will be limited in the surveillance sampling strategy because they provide substantially less information than other animals accompanied by clinical history. Nonetheless, to include representatives from the three most productive surveillance streams, a portion of the maintenance sample will come from animals that are dead with unknown clinical history. These samples will be collected only by FSIS-inspected slaughter facilities and rendering or 3D/4D facilities. Apparently healthy animals are not targeted for surveillance.

Provided that some details of clinical history are known and are supplied under the "clinical signs" section of the data collection form, animals that are dead of unknown cause will be included in surveillance, regardless of sample collection site. The known clinical signs do not need to be recognized as being associated with the cause of death,

and do not need to represent clinical signs consistent with BSE. It is emphasized that efforts for collecting clinical history data will be augmented for Maintenance Surveillance and that samples from animals with known clinical history are much more desirable than those without.

The targeted population for Maintenance Surveillance consists of cattle of any breed that fit one of the following clinical presentation strata:

1. Cattle of any age with CNS signs

This stratum includes cattle exhibiting signs consistent with a central nervous system disorder (including rabies-negative cases from public health laboratories, and FSIS condemns for "CNS signs" or "rabies"). It also includes cattle highly suspicious for BSE as indicated by VS Memo 580.16: 1) cattle affected by illnesses that are refractory to treatment (including anorexia, loss of condition in spite of good appetite, pneumonia, decreased milk yield) and are displaying CNS or behavioral changes that are not of an acute nature (including apprehension, nervousness, excitability, aggression, head shyness, hypermetria, kicking when milked, difficulty in rising, excessive nose scratching, hesitation at gates/barriers); 2) cattle displaying progressive neurological signs that cannot be attributed to infectious illness and are not responsive to treatment.

2. Cattle ≥ 30 months of age[ii] that are condemned during antemortem inspection at slaughter, or are excluded from slaughter due to poor health status (unhealthy or dead due to illness or injury)

This stratum includes cattle that are condemned by FSIS at antemortem inspection for any reason (other than "CNS signs" or "rabies").

Additionally, this stratum includes cattle that are presented to collection sites other than slaughter, are dead or have poor health status, and have known clinical history. An animal's inclusion in surveillance is not limited by the types of clinical signs that are known, or by the presumptive cause(s) of known clinical signs. Cattle that are dead with unknown clinical history, and present to collection sites other than an FSIS-inspected slaughter plant, or rendering or 3D/4D facility, will not be included in surveillance.

The size of the targeted population available to enlisted sample collection sites, and corresponding sample points, have been estimated based on Enhanced Surveillance data (see Table 3.3). The values in Table 3.3 demonstrate that the number of targeted cattle available to enlisted collection sites is sufficient to meet or surpass the projected sampling goal (40,000 samples, 42,857 OIE points, 414,285 analytical points; see section II.D). The methods proposed to conform to the sampling goal are discussed elsewhere. Since sample collection efforts will be adjusted for Maintenance Surveillance, the

[ii] Age of 30 months or older is evidenced by the eruption of the second set of permanent incisors.

precision of these estimates is uncertain. For example, efforts to enlist participation of accredited veterinarians to collect on-farm samples during Enhanced Surveillance varied by state. Therefore, it is possible that when such efforts are equal between states, the number of samples collected on-farm could exceed 22,000. Additionally, FSIS-contracted facilities may have reduced interest in surveillance participation if they are no longer collecting samples other than those provided by FSIS. Animals that were dead with unknown clinical history have been included in the Table 3.3 data, although these animals will not be accepted from certain collection site types during Maintenance Surveillance. However, since efforts during Maintenance Surveillance will be shifted to emphasize the importance of collecting clinical history data, and to emphasize the desirability of samples from animals with known clinical history, the proportion of samples from deadstock with no clinical history is expected to substantially decrease. Therefore, the average point per sample available to surveillance (9.7) has probably been underestimated. The precision of this estimate is uncertain.

Table 3.3. Estimated data resources available to surveillance. Estimates for the number of available targeted animals have been derived from 12 months of Enhanced Surveillance data (December 1, 2004 through November 30, 2005). A sample quota has been allotted to "Rendering or 3D/4D Facilities", however, the sample number estimates for all other collection site types correspond to totals observed during one year of Enhanced Surveillance. Available OIE sample points estimates have been determined as the product of estimated sample number and average points per sample according to collection site type (derived from table 3.2). Focusing sample collection efforts on collection sites which have historically supplied samples with the highest average point value appears to represent a feasible sampling methodology since sample numbers and points estimated to be available to data sources for surveillance (collection sites) exceed sample number and point goals.

Collection Site Type	Estimated Sample Numbers	Estimated OIE Sample Points
On-Farm	22,000	409,200
Veterinary Diagnostic Laboratories	1,600	92,640
Public Health Laboratories	100	36,680
Miscellaneous*	3,500	8,750
Slaughter (FSIS)	6,000	24,600
Facilities contracted to collect samples for FSIS	18,000	73,800
Rendering or 3D/4D Facilities**	5,000	5,000
Total	64,100	620,670 (Average point per sample = 9.7)

*Miscellaneous sample sources include veterinary clinics, livestock markets, and contracted sample collection sites

**The actual number of targeted animals available to this collection site type far exceeds 5,000, however this value represents the maximum number of samples that will be accepted from this collection site type.

Surveillance Streams

Samples meeting the target criteria will be assigned to the surveillance streams described in Article 3.8.4.2 of the OIE Terrestrial Animal Health code. The stream to which a sample is assigned will be based on clinical signs that are provided, sample source, and condemnation code data. Note that samples within the "Routine Slaughter" stream do not meet the targeted sample criteria in Maintenance Surveillance. The OIE surveillance stream categories and the criteria by which Maintenance BSE samples will be assigned to them are listed below.

1. **Clinical suspect** – cattle over 30 months of age displaying behavioral or clinical signs consistent with BSE.

Article 3.8.4.2 of the OIE Terrestrial Animal Health code describes this groups as follows: "Cattle affected by illnesses that are refractory to treatment, and displaying progressive behavioral changes such as excitability, persistent kicking when milked, changes in herd hierarchical status, hesitation at doors, gates and barriers, as well as those displaying progressive neurological signs without signs of infectious illness are candidates for examination. These behavioral changes, being very subtle, are best identified by those who handle animals on a daily basis."

Samples will be assigned to this surveillance stream if they are from cattle in a foreign animal disease investigation for CNS signs, were tested negative for

rabies at a public health or veterinary diagnostic laboratory, had CNS signs or were condemned by FSIS for CNS signs or rabies, or if the likelihood ratio for clinical signs associated with BSE is above an appropriate cutoff value (methods for these determinations are described elsewhere).[46] Most of these samples are anticipated to be derived from cattle that are collected from on-farm. A substantial number will also be contributed by FSIS, veterinary diagnostic laboratories, and public health laboratories.

2. **Casualty slaughter** – cattle over 30 months of age that are non-ambulatory, recumbent, unable to rise or to walk without assistance, sent for emergency slaughter, or condemned at antemortem inspection

Samples will be assigned to this surveillance stream if the likelihood ratio for clinical signs being associated with BSE is below an appropriate cutoff value. Most of these samples are anticipated to be derived from FSIS-inspected slaughter plants. However, a considerable number will be contributed by other data sources such as on-farm and veterinary diagnostic laboratories.

3. **Fallen stock** – cattle over 30 months of age that are found dead on farm, or during transport to or at an abattoir.

Samples will be assigned to this surveillance stream if they are derived from an animal that died and has an unknown clinical history, or if the likelihood ratio for

clinical signs being associated with BSE is below an appropriate cutoff value. These samples will only be accepted from FSIS inspected plants and rendering or 3D/4D facilities, and are anticipated to comprise a small portion of the study population and a negligible portion of total points.

Sample collection sites

The following sites have been selected based on observations during Enhanced Surveillance including average point value per sample, total sample numbers, and relative validity and quality of data collected. Additionally, these collection sites comprise the most geographically representative avenues through which cattle may exit the adult cattle population and be available for sampling. That is, most of these collection site types are accessible to targeted animals regardless of the region where cattle are derived. All animals fitting the targeted population that are presented to these data sources will be sampled for surveillance, except for renderering or 3D/4D facilities where a quota of 5,000 samples has been established. Note that proportional goals for sample numbers have not been applied to each collection site type.

1. **On-Farm**

These samples may be collected by accredited veterinarians, federal or state employees (including animal health technicians), or VS-approved dead stock haulers. Under VS Area Office oversight, sample collectors with other qualifications may be enlisted when resources preclude the participation of aforementioned sample collectors in a given area. Although these samples may

have a higher cost relative to other data sources, they are anticipated to have higher value to surveillance since the accuracy, quantity, and perceived validity of historical clinical signs data is superior relative to other data sources. Additionally, this source is important for capturing clinical suspects that would have not been presented to other data sources.

2. **Veterinary Diagnostic Laboratories**

Cattle submitted for necropsy, or fresh whole brainstem submitted for ancillary diagnostics to veterinary diagnostic laboratories, including those not involved in BSE testing, will be sampled by laboratory personnel. Such samples are usually accompanied by significant historical information pertaining to clinical signs, and thus are of high value to surveillance.

3. **Public Health Laboratories**

All samples from cattle that are rabies suspects and test negative for rabies will be submitted for surveillance by laboratory personnel. All samples derived from this data source can be characterized as clinically suspicious for BSE, and thus are of high value to surveillance.

4. **Miscellaneous**

Miscellaneous sample sources will include veterinary clinics, livestock markets, and contracted sample collection sites where samples and corresponding clinical history information will be collected by veterinary professionals or government

employees. These data sources are important for capturing clinical suspects that do not present to other collection sites.

5. **Slaughter (FSIS)**

All cattle 30 months or older condemned at antemortem inspection, and cattle of any age condemned for "CNS signs" or "rabies", will be sampled by FSIS employees or designated off-site sample collection facilities. Most of these samples are anticipated to represent cattle belonging to the second most valuable surveillance stream ("casualty slaughter").

6. **Facilities contracted to collect samples for FSIS**

Samples derived from animals presented to slaughter and subsequently condemned may be collected by personnel of a contracted rendering or 3D/4D facility, or other APHIS approved facility. Under these circumstances, communication of clinical history and condemnation codes to the contracted facility is imperative.

7. **Rendering or 3D/4D facilities**

In order to represent the "fallen stock" surveillance stream and a wide variety of data sources, 5,000 samples will be collected from targeted cattle presenting to rendering or 3D/4D facilities. A quota is selectively applied to this collection site type since the average point value per sample derived from this source is much

lower than other enlisted collection site types, and since this source services select geographical areas.

Cattle that fit the characteristics of the targeted subpopulation "Cattle of any age with CNS signs" will be sampled for surveillance regardless of whether the site they are presented to has been specifically enlisted by surveillance. It is emphasized that cattle identified with the surveillance criteria of "clinical suspect" will be sampled throughout the surveillance period regardless of the avenue through which they present to surveillance, and regardless of the degree with which sampling goals have been met.

Sampling methods

The purpose of sampling is primarily to detect disease, with intent to maintain confidence in low prevalence and detect upward change in prevalence above 1 case per 1 million adult cattle. The presentation of an animal meeting the targeted criteria (see Population Characteristics, section II.B) to a site or recognized sample collector (see Sample collection sites, section II.C) warrants initiation of data collection.

Initially, as many targeted animals as possible that are presented to eligible collection sites, except rendering and 3D/4D facilities (see above), will be sampled. Therefore, sampling rate corresponds to the rate with which targeted animals present to data sources. Given that the targeted population has been divided into strata based on clinical findings, a stratified sampling strategy will be employed. However, proportional weighting will

not be applied to the strata (i.e. a goal for the proportion of samples that will be derived from each targeted subpopulation will not be established).

Sample Points and Numbers to Meet OIE Surveillance Standards

The OIE BSE surveillance guidelines recommend a target number of surveillance points for Type A surveillance based on the size of a country's cattle population. These points are accrued over 7 consecutive years and are used to evidence validity of the BSE status established by surveillance efforts. For a large cattle population, using the design prevalence of 1 detectable case per 100,000 adult cattle and 95% confidence, 300,000 total points over 7 years, or 42,857 points per year are a requirement under international guidelines for *Controlled Risk* country status and is prerequisite for the U.S. to become a *Negligible Risk* country. If the average sample collected through Maintenance Surveillance provides 9.7 OIE points (see Table 3.3), surveillance efforts would require approximately 4,400 samples per year to meet international standards. The degree of uncertainty associated with the estimated average OIE point value per sample is emphasized since the extent to which the productivity (i.e. average OIE points per sample, total sample numbers) of certain collection site types will persist during Maintenance Surveillance efforts can not be reliably predicted.

Sample Points and Numbers to Maintain Confidence in BSE Prevalence Estimates

In the interest of exceeding international standards, and to maintain confidence in previous BSE prevalence estimates and assurance that BSE prevalence remains low in

U.S. cattle, Maintenance Surveillance methods will adopt a design prevalence of 1 detectable case per 1,000,000 adult cattle.

OIE points have been designed to represent the most conservative scenario of the characteristics of the cattle populations of all member states. However, since the U.S. has relevant data pertaining to the demographic characteristics of its adult cattle population, sample values calculated with the same analytic model used to develop the OIE points (BSurvE) result in more precise point values than the conservative OIE estimates (these points are hereafter referred to as "analytical points").[47] Since each analytical point corresponds to a single random sample, using the Cannon and Roe method,[48] given a population size of 42 million adult cattle, 95% confidence that prevalence is below 1 detectable case per million adult cattle could be achieved by accumulating a total of approximately 2.9 million analytical points over a period of 7 years (414,285 analytical points per year). The average sample collected through BSE surveillance efforts to date has produced approximately 10 analytical points (i.e. roughly 6 million points were accumulated from around 600,000 samples).[41] If this average value remains consistent during Maintenance Surveillance, then surveillance efforts would require approximately 40,000 samples per year to meet this objective. However, given the alterations in sampling strategy adapted for Maintenance Surveillance, the average analytical points per sample is expected to substantially increase. Because of the large sample size collected during Enhanced Surveillance, this goal would be easily met by a small number of samples for the next six years, while obtaining 40,000 samples per year would likely conform to the goal indefinitely.

Sampling rates will be monitored on a monthly basis. If sample numbers evaluated initially by Maintenance Surveillance considerably exceed expectations, sampling strategy can be adjusted to become more prescriptive on the types of samples that will be accepted by the surveillance program.

Although data from Enhanced Surveillance indicate that sample numbers obtained through Maintenance Surveillance are more likely to exceed the sample size goal, in the event that sample numbers fail to meet expectations, efforts for on-farm sample collection could be augmented. Additionally, the sample quota allotted to rendering facilities or 3D/4D facilities could be increased.

Regardless, sample points derived from Enhanced Surveillance have provided a substantial cushion for potential deficiency during the adjustment to Maintenance Surveillance. Since international standards allow points from Enhanced Surveillance to be included in surveillance analysis over a 7 year block of time, there is considerable time to adapt Maintenance Surveillance to precisely match projected surveillance needs with the most cost-efficient methods of sampling.

Study Area Under Surveillance

The data sources for this sampling plan generally represent catchment areas that have been selected to include animals from all sections of the U.S., and comprise geographically unbiased avenues through which cattle may exit production. In

combination, these data sources provide the opportunity for cattle residing in any part of the country or segment of industry to be sampled:

- Slaughter facilities are geographically unbiased because these service every constituent of the production industry. Additionally, western states that practice open range grazing and do not have access to renderers, or may not observe animal deaths, will still ship cull cattle to FSIS inspected facilities in other states.

- On-farm sample collection allows that samples can be collected wherever cattle reside, minimizing geographical bias. APHIS recommends that the enlistment of veterinary professionals and paraprofessionals for on-farm sample collection be consistent across the nation to prevent geographic bias for this data source.

- There are no areas in the nation that cannot submit fresh whole cattle brain to a public health or veterinary diagnostic laboratory.

A precise quantitative approach is difficult to formulate to ensure that the population sampled by surveillance evenly represents the geographical distribution of targeted cattle in the U.S. Results derived from a quantitative analysis of geographical representation would be difficult to interpret since cattle movement in the U.S. is poorly defined, and the location of a sample collection site may not correspond to the location of animal

origination. Additionally, animal origin data is not precise in that it does not necessarily represent the area where the animal was born or resided most its life, and therefore differs from the area where BSE agent transmission would have occurred. Further, statistical comparison of the geographic regions used in the Enhanced Surveillance analysis and the paucity of positive cases provide no evidence that BSE prevalence varies within the U.S. Therefore, a qualitative strategy of analysis based on the epidemiological characteristics of the sub-populations included in the sample as well as origin and collection site data will be employed for maintenance surveillance sampling.

Data Collection Methods

The methods of data collection will be uniform across collection sites and will utilize the structures and processes established in the Enhanced Surveillance program.

Sample data will be collected using the forms employed by Enhanced Surveillance: USDA BSE Surveillance Submission Form and USDA BSE Surveillance Data Collection Form. Forms are completed by the sample collector either by hand (paper forms) or electronically, through the NAHLN website (nahln.aphis.usda.gov/nahln/jsp/login.jsp). In either case, a hard copy of the BSE Surveillance Submission Form must be submitted to the diagnostic laboratory such that samples can be appropriately identified and assigned an accession number, and results can be supplied to the submitter. If samples are not accompanied by the appropriate submission forms with all necessary information, it is the responsibility of the diagnostic laboratories to contact the sample collection site. Samples will be tested only after necessary information is acquired. Diagnostic

laboratories may report collections sites that are repetitively problematic to the appropriate AVIC for correctional efforts. If the collection site is incapable of completing data entry electronically (completed paper forms only), paper copies must also be forwarded to a designated USDA:VS office, or the BSE Helpdesk for data entry. Training of data entry is overseen by AVIC offices and VS regional offices, the discretion of state veterinary offices, and the BSE Help Desk.

The USDA BSE Surveillance Submission Form must be completed for each sample batch for a single collection site and date. This form requests submitter information including collection site type, collection site address, and collector identification. Additionally, a BSE sample ID bar code, duplicate to those on the sample tubes, is supplied on this form for each sample.

The USDA BSE Surveillance Data Collection Form must be completed for each animal sampled. Several parameters are recorded on this form. A "Primary Reason for Submission", is used to reassure the sample collector that the animal being sampled is appropriate for surveillance (i.e. is a member of the targeted population). The form includes fields for owner (animal source) address and contact information, animal characteristics including sex, age, breed, verification that the 2^{nd} incisor is erupted, and country of origin if non-domestic. All types of ID present on the animal has must be provided, including silver tag number, owner ear tag number, vaccination tag number, condemnation tag number, back tag number, bangle tag number, ear tattoo, brands, or microchip. It is imperative that the clinical signs section be completed as thoroughly and

accurately as possible because data from this section of the form are used for allocation of samples into appropriate surveillance streams during data analysis. Note that "dead with unknown clinical history" can only be selected if the animal has been condemned at slaughter, or has been submitted to a rendering or 3D/4D facility, because deadstock will not be accepted from other sample collection site types. If the animal has been condemned by FSIS, the relevant condemnation code must be provided.

Both forms require a referral number, a unique identifier that is used to associate the Submission Form with the Data Collection Form. The format consists of 12 alpha numeric characters. The first two characters are the State's abbreviation, the second three are the collectors initials, the following six are the collection date, and the last character is a letter representing designating number of submission batches (A – first, B – second). For FSIS collections, the state abbreviation is replaced by the FSIS Establishment Number.

Data quality oversight is achieved through evaluating average point per sample scores by region with weekly/monthly reports.

Data relevant to the results of laboratory testing are entered through the NALHN interface by diagnostic laboratory personnel. Results can include any of the following:

- Not Detected – negative by ELISA or IHC

- Not detected, not obex* – negative by ELISA; although the sample appeared to be brainstem, the laboratory technician could not identify the appropriate location of obex for sample testing
- No test* – sample not tested because sample could not be recognized as brainstem by the laboratory technician
- Initial reactor – positive on first screening (ELISA) test
- Inconclusive – positive when screening (ELISA) test is repeated in duplicate
- IHC Inconclusive – sample with equivocal immunohistochemistry results
- Positive – samples positive either by immunohistochemistry or immunoblotting

*For these selections, the reason for the result must be further specified as one of the following:

- Advanced tissue decomposition
- Wrong anatomic location
- Tissue disrupted preventing anatomic orientation

Sample Collection Methods

All animals which befit the targeted population that are identified by collection personnel or presented to collection sites enlisted by surveillance should be sampled. Samples may be collected by authorized federal or state personnel, accredited veterinarians, APHIS-contracted employees, or diagnostic laboratory personnel.

Animal identification items (drawings or digital pictures of brands, removed tattooed hide, ear tags, etc...) should be collected from each animal sampled, bagged, labeled with the sample number, attached to a copy of the USDA BSE Surveillance Submission Form and saved by the sample collector until negative results are received.

Brainstem samples may be collected through the foramen magnum, using a brain spoon or other extraction techniques (such as water extraction, compressed air), after disarticulation of the atlanto-occipital joint. Alternatively, brainstem sample may be collected by dismantling the calvarium (e.g. when retrieving the whole brain for rabies diagnosis). An appropriate brainstem sample includes obex, and is affected with little contamination or postmortem decomposition. Samples that are affected with post-mortem or post-collection decomposition such that they cannot be recognized as brainstem will not be tested by the diagnostic laboratory. Sample collectors should submit samples that have questionable testability and allow laboratory technicians to decide if tissue integrity precludes testing. Diagnostic laboratories will be compensated for efforts related to determining sample testability.

Fresh brainstem samples are individually packaged in plastic tubes that are labeled with a unique BSE sample identification bar code supplied by the USDA. Samples are sent to a NALHN laboratory that is participating in BSE surveillance and will be offering BSE test service the following business day. If samples are received by a diagnostic laboratory on a non-operating weekday, samples will be held under refrigeration by the diagnostic laboratory and subsequently tested on the next operating weekday. If test results are

urgently needed, the AVIC office may dictate that samples be redirected to an operating diagnostic laboratory.

Feedback for poor sample collection technique by collector will be accomplished during reporting of results. For samples that receive the results, "no test" or "not detected, not obex", an informative statement explaining the reasons for these results will be provided. Provision of this statement is the responsibility of the pertinent AVIC. It is the responsibility of the AVIC offices to monitor sample collectors in the relevant area for habitual poor sample collection technique.

Training of sample collectors is completed by AVIC offices and VS regional offices, and the discretion of state veterinary offices.

Sample Chain-of-custody

Samples are enclosed with cool packs in insulated packages, and are shipped by overnight contract delivery service (e.g. Federal Express), same-day courier service, or by hand delivery. A paper copy of each submission form must be submitted to the diagnostic laboratory with the samples. Delivery verification and trouble-shooting is the responsibility of the sample collector. For samples with "inconclusive" test results, all remaining tissue must be immediately forwarded to NVSL (per the most current version of NVSL protocol GPPISOP0029).

Time Intervals

Samples may be compiled prior to submission to the diagnostic laboratory, provided that they are refrigerated. Samples should not be frozen. These should be submitted as soon as possible, but may be pooled for no longer than 7 days. Delivery of samples to diagnostic laboratories should be completed within 24 hours so as to preserve sample integrity. Samples received by diagnostic laboratories should be tested within 24-48 hours for screening tests, or within 7-10 days for immunohistochemistry. The diagnostic laboratory is responsible for entering test results into the NAHLN database and for notifying the sample submitter of test results. This should be completed within 24-48 hours of test completion. The timeline of procedures that occur in response to a positive case are described elsewhere.[42,43]

Data Dissemination

Sample data including test results are maintained in a centralized database following entry through the NAHLN interface. Testing results are available to qualified personnel on the NAHLN website. Diagnostic laboratories must notify the sample submitter of test results either electronically, by phone, or in writing. When samples have been submitted by a facility contracted by FSIS, both FSIS and the contracted submitter must be notified of results. Additional parties, including the AVIC and state veterinary office, may also be notified of results given that an arrangement has been agreed upon between the diagnostic laboratory and additional party. The process for reporting positive test results is described elsewhere.[42,43]

Animal Disposal

Carcasses from negative animals are disposed of in compliance with Federal, State, and local laws. Carcasses and offal from "inconclusive" or positive animals may be disposed of by one of the following: rendering for non-animal feed use by dedicated facilities, burial in a landfill, burial on-farm, alkaline digestion, or incineration. Rendering facilities may refrigerate or freeze carcasses, or may proceed with rendering and hold batches of final products, pending test results. Should a positive animal occur with the latter method, an indemnity would be supplied for the disposed batches of products.

Case Definitions

Clinical Case Definition

Clinical case definitions are not applicable to the present surveillance methods. Because clinical signs have such poor specificity and sensitivity for BSE diagnosis, and because the diagnostic assays used for BSE diagnosis are considered to have near perfect analytic sensitivity and specificity (i.e. cattle with detectable disease are reliably differentiated from cattle without detectable disease), BSE diagnosis is solely based on laboratory criteria. The case definition used for surveillance is the laboratory case definition for BSE as defined by the most current version of NVSL document GPISOP0034. Although the clinical case definition therefore corresponds to the characteristics of the targeted population, clinical signs do not influence the designation of a positive BSE case.

There are no known carrier or reservoir species involved in disease transmission that should be considered for surveillance.

Laboratory Criteria for Diagnosis

The diagnostic strategy implemented for BSE surveillance is described elsewhere (most current versions of NVSL protocols GPPISOP0027 and GPPISOP0034).

Case Classifications

The following classifications are used to imply a level of certainty in a positive diagnosis:

- Initial reactor – positive on first screening test
- Inconclusive – at least one test is positive when screening test is repeated in duplicate
- Positive – samples positive either by immunohistochemistry or immunoblotting

Analysis, reporting, and presentation

Data analysis and interpretation

The NSU is the party primarily responsible for data analysis. Several methods will be used for data analysis:

1. **Monitoring of data quality.** Many mechanisms are in place in the existing BSE database that prevent common data entry errors and that require recording of important sample data. An error-checking routine will be run periodically to identify information that is outside expected values or for key

data that are missing. Samples that do not meet the targeted sampling criteria will be identified, and feedback will be provided to sample collectors that submit non-targeted samples.

2. **Monitoring progress toward national sampling goal.** Reports will address cumulative and monthly totals for the number of samples collected by surveillance. Reports will address the average surveillance points accrued per sample stratified by geographical area and sample collection site type to recognize sample sources that seem to provide deficient data. If significant deficiencies are detected, corrective measures could be pursued during the surveillance period.

3. **Monitoring geographical representativeness.** In the interest of implementation oversight, surveillance productivity by region will be evaluated on a qualitative basis. A quantitative approach will not be conducted since conclusions derived from such an analysis without a fully implemented animal ID system would exceed the precision of available data (see "Study Area Under Surveillance", section II.D).

4. **Determination of OIE surveillance points accumulated on an annual basis.** Accrued OIE surveillance points provide evidence of the validity of surveillance findings and are important for assuring trading partners of BSE status in U.S. cattle. Points per sample are based on the age and surveillance

stream of the sampled animal. Cattle are allotted to surveillance streams based on clinical signs that are provided, sample source, and condemnation code data.

5. **Interpretation of annual surveillance findings.** Analysts will translate surveillance findings into information useful for policy makers in the form of an annual summary report.

Data presentation and reporting

Reports produced by the NSU for the Deputy Administrator of VS and his designates will include monthly reports used for program monitoring and oversight, and an annual summary report analogous to that produced at the conclusion of the Enhanced Surveillance program. The information provided by the annual report may be further tabulated for public consumption at the request of the Deputy Administrator, however monthly reports are anticipated to remain for internal USDA use only. Measures that will be portrayed by the monthly reports, in tabular and graphical format, include monthly and cumulative numbers of targeted samples, non-targeted samples, "not detected, not obex" samples, and total samples stratified by collection site type, surveillance stream, and geographical area. Monthly and cumulative percentages of targeted samples by collection site type, surveillance stream, and geographical area will be presented. The annual summary report is intended to tally surveillance points derived from BSE surveillance efforts over the last 7 years using the OIE Code. Additionally, this report will provide an estimate of BSE prevalence, and/or demonstrate freedom from disease in U.S. cattle.

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CHAPTER 4

Thermal and time-dependent reduction in PrP^{res} immunoreactivity using three immunoassays for transmissible spongiform encephalopathy diagnosis

Abstract

Prions are exceptionally resistant to physical or chemical methods of degradation. Conditions that result in prion destruction under natural conditions or that may be used for environmental decontamination or disposal of biowastes need to be elucidated. It was hypothesized that long-term heating may be effective at destroying prions. The immunodetection of protease-resistant, disease-associated prion protein (PrP^{res}) is an expedient approach to screen physiochemical conditions for potential to destroy prions. The present objective was to characterize PrP^{res} immunodetectability in brain from chronic wasting disease (CWD)-affected elk that was subjected to heat over time. Three commercially available diagnostic assays for CWD demonstrated progressive loss of PrP^{res} immunodetectability when brain homogenates were incubated at 37, 55, and 80° C for 200 days. The rate of PrP^{res} immunoreactivity reduction increased with incubation temperature and was comparatively more substantial when brain homogenates were incubated as 20% solutions in sterile water. When samples were incubated at 55 or 80° C, PrP^{res} immunoreactivity eventually declined below the detection limits of all three assays. Results indicate the potential for environments and biodisposal systems that maintain high temperatures over time to naturally degrade prions. Ultimately, bioassay is necessary to determine whether infectivity is absent from treated samples with depleted, detectable PrP^{res}. Furthermore, findings suggest that PrP^{res} immunoassays may not be

accurate when applied to brain samples that were weakly positive and have had long post-mortem or post-collection intervals, or have been exposed to high temperatures.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of fatal infectious neurodegenerative diseases that include scrapie of sheep and goats, chronic wasting disease (CWD) of free-ranging and captive deer and elk, bovine spongiform encephalopathy (BSE) of cattle, and Creutzfeldt-Jakob disease (CJD) of humans. The BSE agent has been implicated as the cause of variant CJD in humans.[1-3] The potential for foodborne transmission of BSE agent to humans, and cases of CJD occurring in unusually young patients who had consumed venison,[4-6] raise concern for similar zoonosis of CWD agent.[7] The perceived human health risk related to TSEs, especially BSE, has encouraged large economic expenditures for surveillance and containment efforts, and has necessitated the establishment of enhanced food safety measures.

The causative agent of TSEs is believed to be the prion.[8] Little is known about the fate of prions in the environment. Because prions are resistant to several physical and chemical decontamination methods,[9] and one study demonstrated the survival of scrapie agent in the environment for several years,[10] it is generally believed that these agents result in long-term ecological contamination. Prions may be introduced to an environment through shedding of the agent from live hosts, or through decomposition and scavenging of dead hosts. Environmental persistence of prions may provide an avenue for horizontal transmission to susceptible hosts.[11] Contamination of premises with prions is a serious

consequence for herds containing an animal affected with scrapie or CWD and has been suspected in some outbreaks.[12,13] Factors that favor natural prion degradation within the environment need to be elucidated. Accepted methods for prion elimination, including steam-autoclaving at high temperatures, alkaline hydrolysis, treatment with phenolic disinfectant such as LpH, and treatment with sodium hypochlorite, or sodium hydroxide, may not be accessible or practical for disposal of potentially infected animals or for decontaminating the environment.[9,14,15] Alternative methods for disinfecting TSE-contaminated biomaterial or surroundings are needed.

The exact nature of the prion is undetermined, however, it is at least partially comprised of a proteinase-resistant, disease-associated isoform of host-encoded prion protein (PrP^{res}).[8,16] Prions can be demonstrated by immunodetection of PrP^{res} or by animal bioassay. While bioassay is ultimately needed to determine if materials suspected to contain prions are infectious, laboratory animal welfare concerns and the time and expense needed to complete such studies limits its application for screening decontamination protocols.

We hypothesize that over a relatively long period of time, temperature-dependent degradation of prions is more significant than previously considered. Using a Western blot, an initial study demonstrated substantial temperature-dependent, progressive loss of detectable PrP^{res} in brain samples from CWD-affected cervids that were incubated at 37, 55, and 80° C for up to 90 days.[17] Since PrP^{res} detection has been demonstrated to correlate with infectivity,[18] and since modifications to structure or conformation of PrP^{res}

have been associated with reduction in infectivity,[16,19-21] evaluation of PrP^{res} detectability is a convenient approach to screen for conditions that may favor prion destruction prior to conducting bioassay. To substantiate prion destruction, bioassay is needed. However, prior to undertaking the expense and the responsibilities of designing useful animal experiments, alternative explanations of the the initial findings that are unrelated to PrP^{res} destruction and loss of infectivity need to be explored and ruled out. These include selective epitope loss or masking, formation of non-mobile aggregates, and increased sensitivity to proteinase K (PK) use in the test protocol.

The objective of the present study was to demonstrate the consistency of *in vitro*, heat-dependent loss of PrP^{res} immunoreactivity in brain samples from CWD-affected elk, by using 2 EIAs in addition to the Western blot. Each of these PrP^{res} detection systems use different approaches for selective PrP^{res} identification and employ unique antibodies. Here we demonstrate consistent progressive reduction and eventual loss of detectable PrP^{res} in brain samples similarly treated with heated incubation for up to 200 days, and rule out several explanations for the phenomenon.

Materials and Methods

Animals

Elk 1, an adult male, was found dead in a US National Park in the Rocky Mountains. Extensive accumulation of PrP was identified in the retropharyngeal lymph node, tonsil, and nuclei of the obex of the medulla oblongata using immunohistochemistry. The brainstem was affected with moderate spongiform degeneration.

Elk 2, an adult female in poor body condition, died in a US National Park in the Rocky Mountains after being observed with a braced stance and lowered head. Using immunohistochemistry, extensive accumulation of PrP was detected in the retropharyngeal lymph node and nuclei of the obex of the medulla oblongata. The brainstem was affected with severe spongiform degeneration.

Incubation

Brain tissue from the two elk was obtained at necropsy. Caudal brainstems were homogenized separately using a homogenizer[c] and stored at -70° C. 0.5-5 grams of thawed pure brain homogenate were placed in sealed 2-5 ml microcentrifuge tubes. Homogenates from both elk were incubated in replicate trials at 55° C and homogenates from elk 1 were additionally incubated at 37 and 80° C (Table 4.1). At day 0 (un-incubated control), and after approximately 16, 30, 60, 90 and 200 days of incubation, aliquots were removed from homogenates to be evaluated for PrP^{res} detectability using three assays. For each elk, incubation trials at 55 C were performed in duplicate; one homogenate was incubated as whole brain, whereas another was incubated as a 20% concentration of brain in sterile protease-free water. Five replicate incubation trials were completed for elk 1, and 2 were completed at 55° C for elk 2.

[c] Prionics FastPrep Homogenizer, Prionics AG, Zurich, Switzerland

PrP^{res} Immunodetection

Unincubated brain homogenates from both elk contained abundant PrP^{res}. When evaluated with the Western blot, undiluted samples lost lane and band definition because of the abundance of PrP^{res}. When evaluated with the EIAs, OD values were maximized outside the linear range of enzyme detection kinetics. Therefore, control (un-incubated) and incubated samples were diluted differentially for each elk and for each PrP^{res} detection system (as described below) such that the effect of incubation conditions on PrP^{res} immunodetectibility could be accurately evaluated. Since the Western blot and EIA1 utilize PK digestion, dilutions were performed after the PK digestion in order to conform to standard assay conditions.

For the Western blot, control (un-incubated) and incubated brain homogenates were diluted to 10% solutions with 1X kit homogenization buffer (final concentration being equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions) and then digested with PK per manufacturer's protocol (10 ul/100 ul digestion buffer and 1.8 Units/ml PK at 48° C for 40 minutes). Control (un-incubated) and incubated brain homogenates were further diluted at 1/6 (elk 1) and 1/2 (elk 2) with sterile protease-free water following PK digestion. These dilution levels are those which produced the strongest signal without impeding protein bands within adjacent lanes and were below the maximum detection limit of the densitometer. Homogenates were mixed 1:1 with SDS sample buffer prior to electrophoresis. To complete the Western blot, a commercially available kit[d] was used per manufacturer's protocol. PrP^{res} signal intensity

[d] Prionics®-Check WESTERN, Prionics AG, Zurich, Switzerland

was estimated using densitometric analysis of all three CWD-specific PrP^{res} bands.[e] Percent PrP^{res} signal reduction was estimated by comparing intensity of PrP^{res} immunoreactivity between control (un-incubated) homogenates and incubated homogenates at each time-point. For incubation trials performed at 55° C, the mean percent PrP^{res} signal reduction was calculated for each elk.

EIA 1[f] is a sandwich ELISA that uses two monoclonal antibodies and, similar to the Western blot, requires a PK digestion step. For EIA 1, PK digestion was performed on incubated samples according to manufacturer's protocol. Following PK digestion, control (un-incubated) and incubated brain homogenates were diluted at 1/20 (elk 1) and 1/137 (elk 2) with bovine brain homogenate. These dilution levels were those that produced the most repeatable OD values within the linear range of enzyme detection substrates. The final concentrations were equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions. Brain homogenate was used for dilution in order to maintain a consistent test matrix. Bovine brain tissue was negative for BSE by all tests used by the present study. Prior to testing, each sample was prepared as an 11% brain homogenate solution in 5% glucose (kit homogenization buffer). The remaining sample preparation was performed according to manufacturer's protocol.

EIA 2[g] is an antigen capture ELISA that uses a single monoclonal antibody. This second generation, conformation-dependent assay utilizes the differential binding of aggregated

[e] ChemiImager IS-5500, Alpha Innotech Corporation, San Leandro, CA
[f] Bio-Rad TeSeE®, BioRad, Paris, France
[g] IDEXX HerdChek® BSE antigen test kit, IDEXX Laboratories Inc., Westbrook, ME

PrP^{res} to Seprion® affinity ligands, and thus is not dependent on PK digestion.[17] EIA 2 was completed per manufacturer's protocol. Prior to testing, each sample was prepared as an 11% brain homogenate solution in sterile protease free water. Control (un-incubated) and incubated brain homogenates were diluted at 1/261 (elk 1), or 1/137 (elk 2) with 11% negative bovine brain homogenate. As for EIA 1, these dilution levels were those that produced the most repeatable OD values within the linear range of enzyme detection substrates and the final concentrations were equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions.

For each EIA, OD values were used to represent PrP^{res} signal intensity. Percent PrP^{res} signal reduction was estimated by comparing PrP^{res} signal intensity between control (un-incubated) homogenate and incubated homogenate at each time-point. In addition, for incubation trials performed at 55° C, the mean percent PrP^{res} signal reduction was calculated for each elk. Positive or negative test results were determined by comparing a sample's OD value to OD cut-off values that are established for each test run using control materials provided by the test kits.

Results

Prior to incubation (day 0), homogenized brain tissue from both elk was positive for CWD by Western blot, as indicated by the presence of three distinct protein bands which represent glycosylated forms of truncated PrP^{res}. Likewise, when using either EIA, unincubated, homogenized brain tissue from both elk was consistently positive for CWD as indicated by an OD value for PrP^{res} immunoreactivity that exceeded cut-off OD values.

PrP^{res} immunoreactivity as indicated by each detection system was considerably reduced over time in samples from Elk 1 that were incubated at 37, 55, and 80° C (Fig. 4.1 and 4.2). At all temperatures, Western blots failed to produce PrP^{res} bands when applied to tissues incubated for 200 days. Furthermore, PrP^{res} bands were not evident in Western blots of samples that were incubated at 55 and 80° C for 200 days and were not diluted following incubation and PK digestion (Fig. 4.3). When samples were tested with EIA 1, OD values for PrP^{res} immunoreactivity were reduced below the cut-off for a positive result after incubation at 80° C for 30 days. Although OD values of samples incubated at 37 and 55° C were substantially reduced over time, the OD values remained above the cut-off value after incubation for 200 days. When samples were tested with EIA 2, OD values for PrP^{res} immunoreactivity were reduced below the cut-off for a positive result after incubation at 80° C for 16 days, or 37 or 55° C for 200 days. The rate of PrP^{res} immunoreactivity loss as demonstrated by each detection system increased with incubation temperature. PrP^{res} immunoreactivity exhibited by all detection systems for the sample incubated at 80° C was greater than 80% reduced by day 30 and greater than 95% reduced by day 60. PrP^{res} immunoreactivity as evidenced by all detection systems was substantially reduced over time as a result of incubation at 37 and 55° C, however, the rate of immunoreactivity reduction was less notable when samples were analyzed with EIA 1 versus the other 2 detection systems.

To more precisely characterize the effect of heated incubation on PrP^{res} detectability, multiple incubation trials were completed at 55° C (a temperature relevant to mortality

composting) using brain tissue from both elk. A similar loss in PrP^{res} immunoreactivity was demonstrated over time when evaluating tissue from both elk with each detection system (Fig. 4.4). Further, the rate of PrP^{res} immunoreactivity loss was greater for samples that incubated as 20% aqueous solutions. Homogenates incubated as whole brain from both elk evaluated with the Western blot produced PrP^{res} bands that were variably noticeable after 90 days of incubation, and were always diminished after 200 days of incubation. Whereas most homogenates incubated as 20% aqueous solutions from both elk evaluated with the Western blot were devoid of PrP^{res} bands following 90 days of incubation. When homogenates incubated as whole brain were evaluated with EIA 1, OD values of samples from elk 1 were inconsistently reduced below the cut-off for a positive result following 90 to 200 days of incubation, and were reduced below the cut-off in all samples from elk 2 after 16 days of incubation. OD values of homogenates incubated as 20% aqueous solutions from elk 1 were inconsistently below the cut-off after 200 days of incubation. OD values of homogenates incubated as 20% aqueous solutions from elk 2 were inconsistently below the cut-off after 16 days of incubation, but were always below the cut-off after 60 days. When homogenates incubated as whole brain of both elk were evaluated with EIA 2, OD values were inconsistently reduced below the cut-off for a positive result on the 90^{th} day of incubation and were always below the cut-off after 200 days of incubation. OD values were below the cut-off in all homogenates incubated as 20% aqeous solutions from both elk that were incubated for 90 days and evaluated with EIA 2.

Discussion

Prions are resistant to most conventional decontamination procedures. Methods that have been demonstrated to inactivate prions are often times not practical, available, or cost-effective for disposal of biowastes from TSE-infected animals. Novel disposal and decontamination techniques that prevent or eliminate environmental prion contamination are needed. High-temperature autoclaving has demonstrated inactivation of prions.[9] Should prion decomposition also occur at a slower rate under more moderate heat conditions, there is potential for prion degradation by biowaste disposal systems that maintain temperature for sustained time periods, such as livestock mortality composting,. Using Western blot, a recent study demonstrated loss or reduction of immunodetectable PrP^{res} in tissues from scrapie-affected sheep that were composted for up to 148 days at temperatures periodically above 60° C.[22] The present study reveals a similar reduction in detectable PrP^{res} that was a result of exposure to heat alone, without the presence of complex proteolytic systems maintained by microbial consortia.

Using a Western blot test system, we previously demonstrated progressive loss of detectable PrP^{res} from homogenized brain samples of CWD-affected cervids that were incubated at 37, 55, and 80° C for up to 90 days.[17] The present study substantiates these findings using two additional, dissimilar PrP^{res} detection systems and rules out several trivial explanations for the PrP^{res} disappearance. Each detection system displayed progressive loss of PrP^{res} immunoreactivity over time, notable even on the shortest incubation increment (16 days), regardless of incubation temperature. Furthermore, the rate of PrP^{res} immunoreactivity loss increased with incubation temperature. Over time,

immunoreactivity was observed to decline below the detection limits of all three detection systems when samples were incubated at 55 or 80° C, and declined below the detection limits of the Western blot and EIA 2 when incubated at 37° C. At 55° C, all PrP^{res} detection systems demonstrated that samples incubated at 55° C as 20% aqueous solutions had accelerated loss of detectable PrP^{res} relative to samples that were incubated without dilution.

The use of PrP^{res} detection systems with differential methods for selective PrP^{res} identification was needed to clarify initial findings, as diminished PrP^{res} immunodetection can occur without PrP^{res} degradation. For example, selective epitope loss or masking may preclude immunodetection of PrP^{res}. This seems an unlikely explanation for the present results given that immunodetection was similarly influenced by experimental conditions when using detection systems employing different antibodies. Second, the formation of oligomeric PrP^{res} aggregates may reduce the amount of antigen that is available to immunodetection systems. While this occurrence could provide an explanation for reduction in PrP^{res} immunoreactivity evidenced by Western blot, particularly for samples that have high molecular weight smears of immunoreactive material, aggregate formation should not affect the immunodetection of either EIA used by the present study. Finally, experimental conditions may have increased susceptibility of PrP^{res} to PK. However, this is unlikely as EIA 2 does not use a PK digestion step, and as PrP^{res} immunoreactivity was similarly reduced in non-digested samples evaluated with the Western blot (data not shown). For these reasons, the authors assume that loss of

PrP^{res} immunoreactivity represents PrP^{res} degradation. Further study is needed to elucidate the mechanisms potentially accountable for PrP^{res} degradation.

Prior to testing, it was necessary to differentially dilute brain for each elk and PrP^{res} detection system; therefore, direct comparisons regarding the performance of each assay under the experimental conditions could not be made. However, diagnosticians using PrP^{res} detection systems for CWD diagnosis should be aware that negative test results may have questionable accuracy when derived from brain samples which were subjected to heat over time. This is especially a concern for animals that are in a stage of disease where PrP^{res} accumulation is marginally above a test's detection limits. If exposure conditions are associated with severe brain tissue decomposition, the validity of a negative test result is further jeopardized by the inability to properly identify the appropriate sub-gross anatomic site for testing.

The present study used PrP^{res} immunodetection as a practical approach for assessing a potential prion-degradation effect and ruling out various confounding phenomena that could result in loss of PrP^{res} signal. Such an approach is a necessary precursor to meaningful animal bioassays. We speculate that the time and temperature-dependent reduction in PrP^{res} immunodetection suggests that such experimental conditions could reduce infectivity. Given the present findings, bioassay is warranted to confirm loss of infectivity from samples that are depleted of detectable PrP^{res}. If reduction of infectivity can be demonstrated, it is possible that natural, heat-producing microbial systems can be developed and exploited to decontaminate prion-containing materials.

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Table 4.1. Number of separate incubation trials performed at three different temperatures for each elk. For each incubation trial, incubating brain homogenates were tested using 3 detection systems at 5 time-points over a period of 200 days and were compared to non-incubated control brain homogenate from the respective elk.

	37° C	55° C[+]	80° C
Elk 1	1	5	1
Elk 2	*	2	*

[+]Incubation trials were performed in replicate at 55° C; 5 brain homogenates from elk 1 were incubated as whole brain and 5 were incubated as 20% concentrations of brain in sterile protease-free water. Likewise, 2 brain homogenates from elk 2 were incubated as whole brain and 2 were incubated as 20% concentrations of brain in sterile protease-free water.

*No incubation trials were performed at these temperatures using tissues from elk 2.

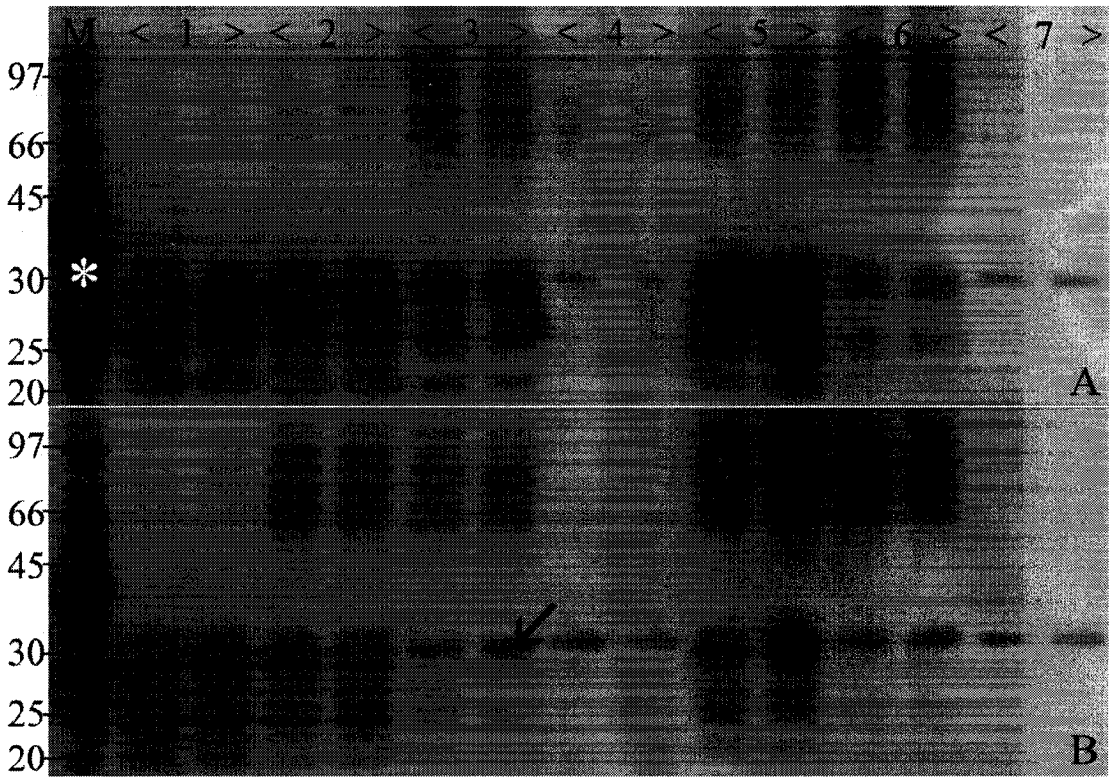

Figure 4.1
Western blots of homogenized brain tissue from elk 1 incubated for 0 to 200 days at 37, 55, and 80° C, and diluted 1/6 in sterile protease free water prior to testing. A = sample incubated at 37° C. B = sample incubated at 55° C. C = sample incubated at 80° C. Numbers at the top of lanes indicate days of incubation. M denotes lane containing molecular weight markers (kDa) and non-protease-truncated PrP (homogenous band spread between 25-35 kDa labeled with asterisk). Samples positive for CWD contain 3 distinct bands (white arrows) starting at 30 kDa that represent glycosylated forms of PrP^{res}. The bands at 31 kDa (black arrow) result from nonspecific binding of the secondary antibody to proteinase K. Note progressive loss of intensity for CWD-specific immunoreactivity in all samples over time.

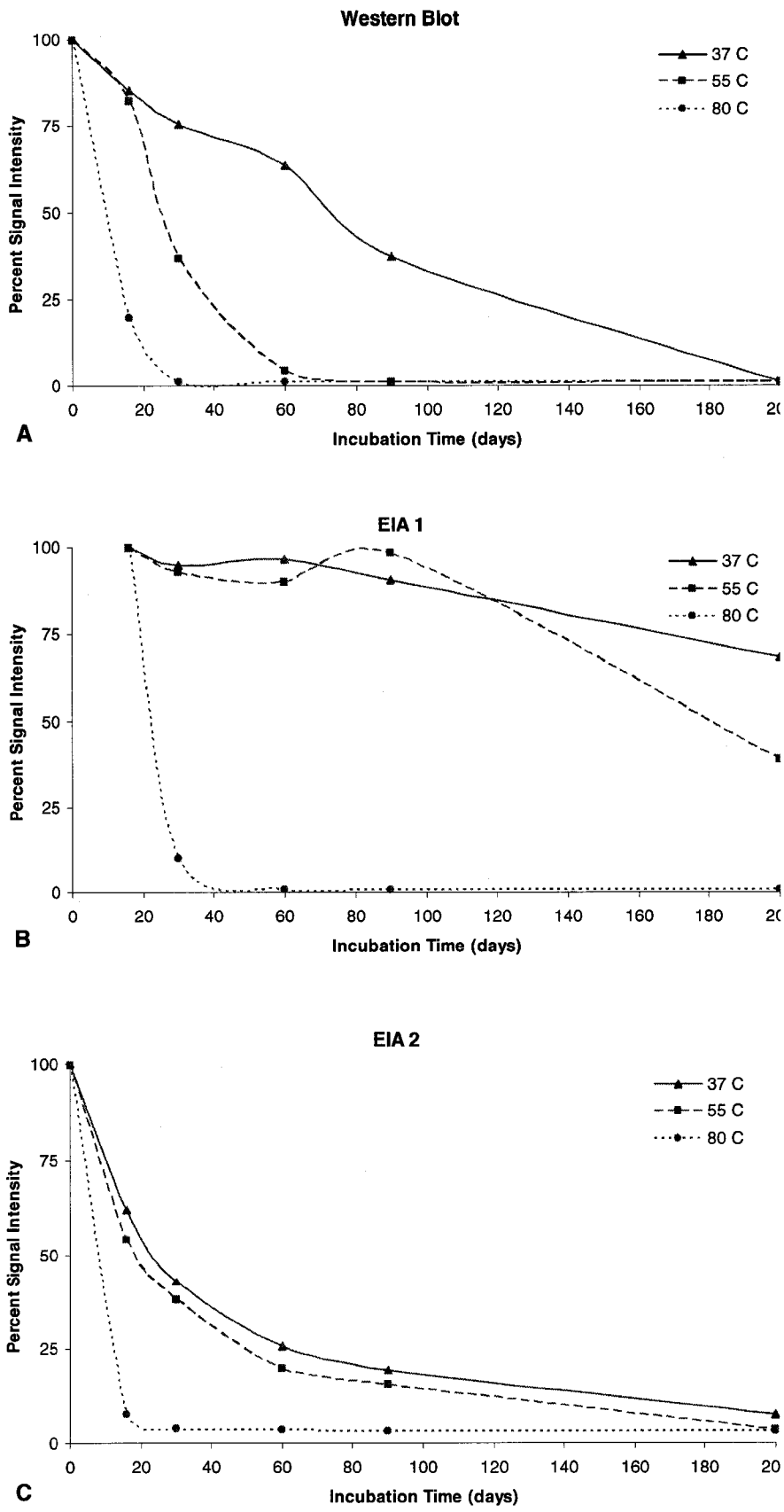

Figure 4.2
Percent PrP^{res} signal reduction as detected by 3 assays when applied to homogenized brain tissue from elk 1 incubated for 0 to 200 days at 37, 55, and 80° C. Signal loss reflected by panel A corresponds to reduction in band density as determined with densitometry applied to the Western blot portrayed in Figure 1. Signal loss displayed in panels B and C represents reduction in optical density values. Incubated brain homogenates were differentially diluted for each assay prior to testing: WB – 1/6 in sterile protease-free water; EIA 1 – 1/20 in BSE-negative bovine brain; EIA 2 – 1/261 in BSE-negative bovine brain.

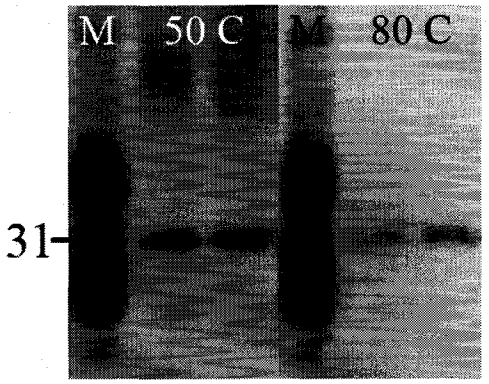

Figure 4.3
Western blots of homogenized brain tissue from elk 1 incubated for 0 to 200 days at 55 and 80° C. Homogenates were not further diluted prior to testing. M denotes lane containing molecular weight markers (kDa) and non-protease-truncated PrP. Note that both samples lack CWD-specific immunoreactivity.

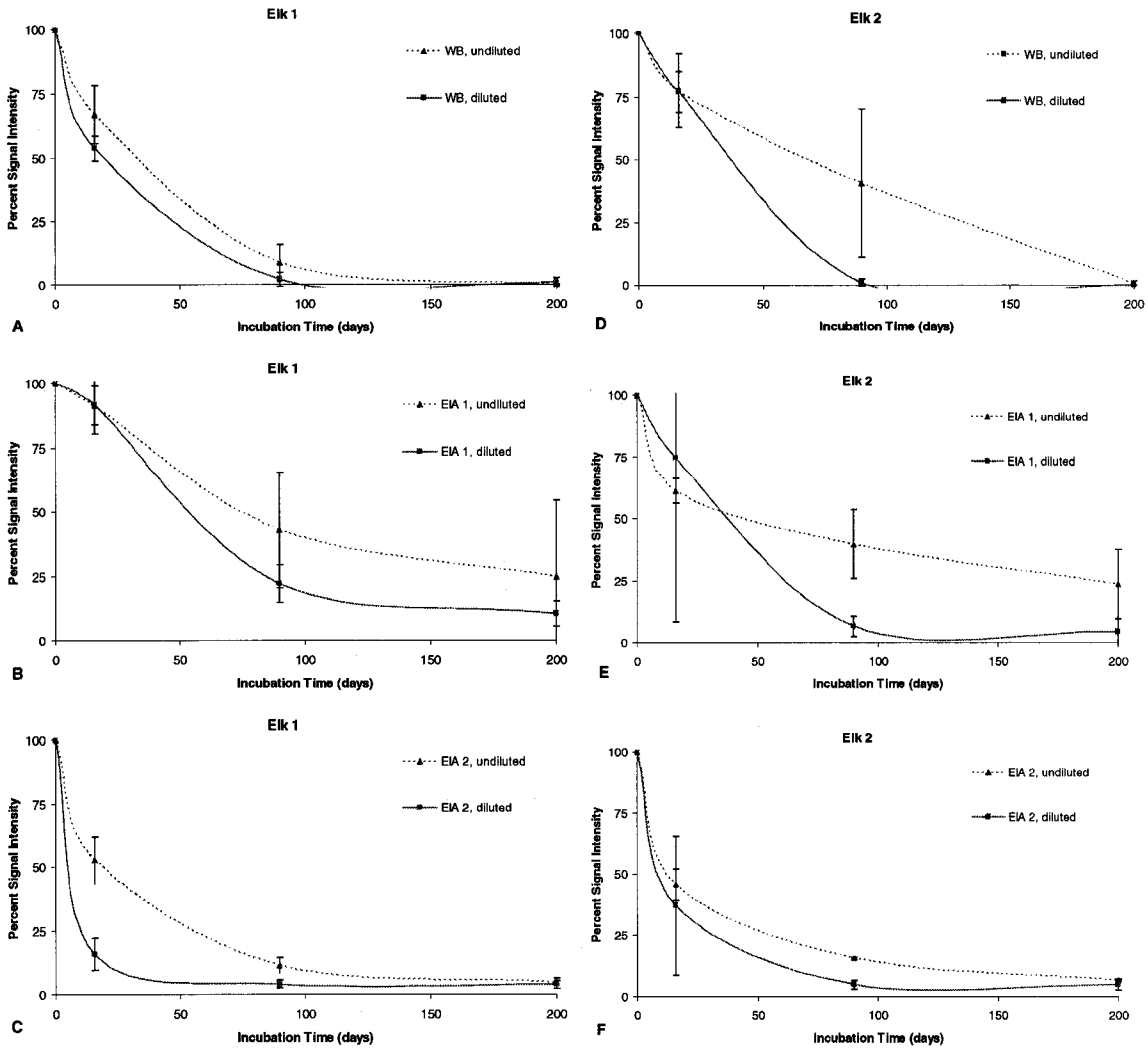

Figure 4.4

Mean percent PrP^{res} signal reduction as detected by Western blot and immunosorbent assays when applied to homogenized brain tissue from elk 1 and elk 2 incubated for 0 to 200 days at 55° C. Signal intensity loss represents reduction in band density as determined with densitometry (immunoblots) or reduction in optical density values (immunosorbent assays). Brain homogenates were incubated as whole brain, or as 20% aqueous solutions. After incubation, homogenates were differentially diluted for each assay prior to testing: WB – 1/6 (elk 1), 1/2 (elk 2) in sterile protease-free water; EIA 1 – 1/20 (elk 1), 1/137 (elk 2) in BSE-negative bovine brain; EIA 2 – 1/261 (elk 1), 1/137 (elk 2) in BSE-negative bovine brain. Data portrayed for elk 1 represent a mean of 5 trials. Data presented for elk 2 represent a mean of 2 trials.

CHAPTER 5

Evaluation of immunohistochemical prion protein detection in recto-anal mucosa-associated lymphoid tissue for scrapie diagnosis in United States sheep

Abstract

In the United States (U.S.), scrapie diagnosis is usually achieved by applying immunohistochemistry (IHC) for disease-associated prion protein (PrP^{res}) to tissues collected postmortem, including obex, retropharyngeal lymph node, and palatine tonsil. Although PrP^{res} IHC applied to third eyelid biopsy allows scrapie diagnosis in live animals, its application has been limited by comparatively lower sensitivity, high frequency of inconclusive (ISF) test results, and the limited amount of tissue available for repeat testing. The objective of the present study was to determine the suitability and estimate the sensitivity of PrP^{res} IHC applied to recto-anal mucosa associated lymphoid tissue (RAMALT) biopsy for scrapie diagnosis in sheep. 532 sheep considered to be high-risk for scrapie from 60 scrapie-exposed flocks were enrolled in the study. Biopsy-related complications were observed in 3 sheep. 90 sheep were designated as scrapie-confirmed by the reference test (PrP^{res} IHC applied to obex and retropharyngeal lymph node and/or tonsil in parallel). The sensitivity of RAMALT biopsy PrP^{res} IHC ranged from 87.5-89.3%. There was no significant difference in sensitivity detected between 3 RAMALT biopsy sites. PrP^{res} IHC applied to right and left ventral RAMALT biopsies in parallel (91.7%) was significantly more sensitive than right and left third eyelids in parallel (86.1%, $p < 0.05$). The proportion of ISF results derived from testing two RAMALT biopsies simultaneously (14.1%) was lower than those derived from testing two third eyelid biopsies simultaneously (22.5, $p < 0.05$). RAMALT ISF results were

usually derived from biopsies that were inappropriately collected from an area caudal to the recto-anal interface. The use of PrP^{res} IHC applied to RAMALT biopsies for scrapie diagnosis in live high-risk sheep is expected to improve the efficiency and success of the U.S. National Scrapie Eradication Program.

Introduction

Scrapie is an invariably fatal infectious disease of sheep and goats that is characterized by slowly-progressive neurologic dysfunction and loss of condition. It is a member of the group of neurodegenerative diseases termed transmissible spongiform encephalopathies (TSEs). Public sensitivity to TSEs, especially those existing in food-producing animals, has followed the emergence of bovine spongiform encephalopathy (BSE),[1] and the recognition of BSE-agent as the cause of variant Creutzfeldt-Jakob disease in humans.[2-4] The origin of BSE is unknown, but one hypothesized surmises that its origination involved scrapie-agent crossing the species barrier when meat and bone meal derived from scrapie-infected sheep was fed to cattle.[5] Public awareness of scrapie has been recently elevated as disease caused by BSE-agent that is difficult to clinically or pathologically distinguish from scrapie has naturally occurred in a goat,[6] and has been experimentally demonstrated in sheep.[7,8] The presence of scrapie in the U.S. has troubled the sheep and goat industry as a result of disease-associated production losses, elevated costs of animal disposal in conjunction with reduced value of ruminant-derived meat and bone meal, and export restrictions of live sheep and goats and certain ruminant-derived

products. It follows that the National Scrapie Eradication Program (NSEP) was founded in 2001 to eradicate scrapie from the U.S. sheep and goat population.[9]

Scrapie surveillance activities in the U.S. include regulatory scrapie slaughter surveillance (RSSS), a national scrapie flock certification program,[10] and testing of clinical suspects and rabies-negative sheep and goats at the discretion veterinarians and diagnostic laboratories. When scrapie surveillance identifies a scrapie-positive animal, field investigations are completed by the NSEP to identify epidemiologically-related flocks that may have been exposed to scrapie-agent. The NSEP conducts testing of a potentially exposed flock to determine its scrapie status and to subsequently direct interventions aimed at reducing the prevalence and transmission of scrapie. Testing and culling efforts are directed at "high-risk" subpopulations believed to have a higher prevalence of, and therefore probability for, scrapie relative to the entire sheep population. Characteristics that define "high-risk" animals have included 1) Demonstration of clinical signs consistent with scrapie; 2) Belonging to the contemporary lambing group of a scrapie-positive ewe;[11] 3) Genetic susceptibility to scrapie defined by certain polymorphisms of the host prion protein gene (PNRP), including homozygous for alanine at codon 136 and heterozygous for glutamine and arginine at codon 171 ($AA_{136}QR_{171}$), heterozygous for alanine and valine at codon 136 and heterozygous for glutamine and arginine at codon 171 ($AV_{136}QR_{171}$), and homozygous for glutamine at codon 171 (QQ_{171});[12-14] and 4) Non-white-faced breed.[14,15] Testing of genetically susceptible or non-white-faced animals is usually limited to females aged 14 months or older because detectable disease is uncommon in young

animals,[16] and because females are believed to be primarily accountable for transmission of disease.[11,17,18]

Presently, the diagnostic approach used by U.S. scrapie surveillance and the NSEP involves examining certain tissues with immunohistochemistry (IHC) to identify the accumulation of disease-associated protease-resistant prion protein (PrP^{res}). Tissues that are typically examined using PrP^{res} IHC are collected postmortem and include obex, tonsil, and retropharyngeal lymph node. This diagnostic approach is capable of identifying infected animals prior to the onset of clinical signs,[19] and is considered to have near perfect diagnostic sensitivity when testing animals with clinical disease.[20]

The lack of diagnostic assays for scrapie that can be used on tissues collected from live animals constrains the effectiveness of scrapie control programs. By using antemortem rather than postmortem testing, disease can be detected within a flock at an earlier time-point, thereby expediting the placement of disease mitigations and reducing continued disease transmission. A reliable antemortem test would reduce the need for indemnification of high-risk animals for testing purposes. The NSEP has used PrP^{res} IHC applied to biopsies of third eyelid lymphoid tissue for antemortem diagnosis of subclinically-affected animals. However, relative to tonsil and lymph node, the quantity of lymphoid follicles is limited in third eyelid biopsies and a large proportion of biopsies contain insufficient lymphoid tissue for evaluation.[21] Furthermore, the test is fairly insensitive when compared to PrP^{res} IHC applied to obex.[22] Therefore, the presence of

scrapie in a flock with negative or inconclusive third eyelid testing cannot be completely ruled out without confirmatory postmortem testing.

In contrast to the third eyelid, rectal mucosa contains plentiful lymphoid tissue and is conveniently accessible for biopsy of live sheep. The quantity of rectoanal mucosa-associated lymphoid tissue (RAMALT) is such that multiple or serial tests of a single animal can easily be performed. Recent studies have demonstrated that sheep infected experimentally or naturally with scrapie-agent accumulate PrP^{res} within RAMALT at a time-point of infection similar to other lymphoid tissues.[23,24] Furthermore, biopsied animals appear to have little post-procedural discomfort, even without anesthesia, and healing of the biopsy site is usually uncomplicated.[24,25]

The purpose of the present study was to determine whether performing RAMALT biopsy under field conditions is a suitable approach to test sheep for scrapie in the U.S. In addition, the present study was conducted to estimate the sensitivity of PrP^{res} IHC applied to RAMALT biopsy relative to PrP^{res} IHC applied to obex, tonsil, and retropharyngeal lymph node in parallel, and to determine the number of lymphoid follicles a biopsy should contain in order to detect disease.

Mterials and Methods

Animals

Animals enrolled in the study were sheep 4 months of age or older, that were clinically suspicious for scrapie, or were designated as high-risk for scrapie and were from scrapie-exposed flocks. Clinically suspicious animals were those that were determined by veterinarians to have clinical signs possibly consistent with scrapie. High-risk animals were those that: 1) Belonged to the contemporary lambing group of a scrapie-positive ewe; OR 2) Were geneticly susceptibile to scrapie as indicated by the presence of one of the following PNRP genotypes: $AA_{136}QR_{171}$, $AV_{136}QR_{171}$, QQ_{171}.

Tissue sampling and data collection for each sheep was conducted by the veterinarians and veterinary technicians responsible for implementation of the NSEP in that sheep's geographical region of residence.

Data Collection

Veterinarians completed standard regulatory forms (Veterinary Services' 5-29 and/or 10-4 forms) for each sampled sheep. These forms were used to collect individual sheep information including animal identification numbers, flock identification number, state of residence, sex, breed, and age. To complete these forms, veterinarians used clinical observations and historical information obtained from flock records and owner correspondence. In addition, a supplemental data form was completed to collect the following information for each sheep: PNRP genotype at codons 136 and 171, presence

and character of clinical signs consistent with scrapie, and any complications that developed following the RAMALT biopsy procedure. PNRP genotype was determined by examining records maintained by the NSEP.

Tissue Sampling

The following tissues were collected for immunohistological evaluation: 1). RAMALT samples taken from the right ventral (RV; i.e. 3-5 o'clock area), left ventral (LV; i.e. 7-9 o'clock area), and left dorsal (LD; i.e. 10-11 o'clock area) positions; 2) third eyelid lymphoid tissue biopsies, one taken from each eye; 3) medulla oblongata at the level of the obex; 4) retropharyngeal lymph node; 5) palatine tonsil. RV and LV RAMALT biopsies and third eyelid biopsies were sampled from live sheep. LD RAMALT samples, obex, retropharyngeal lymph node, and palatine tonsil were collected postmortem.

RAMALT is comprised of lymphoid follicles that circumferentially occupy the mucosa of the anal mucocutaneous junction and proceed rostrally, occupying the most aboral 1-2 cm of rectal mucosa.[26] Prior to collecting RAMALT biopsies, ophthalmic 0.5% proparacaine hydrochloride or 2% lidocaine gel was administered topically to the junction of non-haired skin of the anus and rectal mucosa (anal mucocutaneous junction). Biopsies were first collected from the LV position, and were subsequently collected from the RV position. Abaxial or lateral pressure was placed on the perianal area or a rectal speculum was used to visualize rectal mucosa. To aid visualization, headlamps or chemical restraint were sometimes used. Toothed forceps were used to grasp the rectal mucosa at the mucocutaneous junction and retract caudally. Scissors were used to

establish a cut in the mucosa at the anal mucocutaneous junction, and to extend the biopsy 1 cm cranially (orally) by superficially undermining mucosa away from the underlying connective tissue, producing a 1.5 cm x 2 cm oblong biopsy. The biopsy was rolled out flat, mucosal side down, within a sponge-lined histology cassette.

Third eyelid biopsies were collected as previously described.[21] Briefly, ophthalmic 0.5% proparacaine hydrochloride was applied topically. The third eyelid was retracted using toothed forceps. Lymphoid tissue was collected from the bulbar surface of the third eyelid using Metzenbaum scissors. Third eyelid biopsies were laid flat with bulbar surface facing down within a sponge-lined cassette.

Sheep were humanely euthanized within 3 weeks of collecting antemortem tissue samples. Postmortem tissue collection was commenced within 24 hours of euthanasia.

The caudal rectum was removed, opened longitudinally, and examined for the presence of any gross abnormalities, including, but not limited to, rectal prolapse, cellulitis, rectal stricture, hemorrhage, perirectal abscess, and peritonitis. Samples of rectal mucosa resembling those collected from live animals were collected from the LD position.

PrP^{res} IHC

All tissues collected for immunohistochemical evaluation were immersed in 10% neutral buffered formalin and were fixed for at least 48 hours. Formalin-fixed tissues were processed conventionally. Care was taken to embed RAMALT and third eyelid biopsies

flat within paraffin wax, such that the biopsy side comprised of rectal mucosa or bulbar conjunctiva was positioned in the same plane as the surface of the block. Immunohistochemical staining was conducted at the NVSL; the procedure was similar for each tissue type. Paraffin-embedded tissue samples were sectioned at 3 to 5 μm, mounted on positively-charged glass slides, and air-dried overnight. Slides were rehydrated with xylene and graded alcohols. For antigen retrieval, sections were first treated with 95-98% formic acid for 5 minutes and then washed in Tris buffer. Next, slides were placed in modified citrate buffer (Target Retrieval Solution, Dako Corporation, Carpinteria, CA) and were autoclaved at 120° C for 20 minutes in a medical pressure cooker (BioCare Medical Decloaking Chamber, BioCare Medical, Walnut Creek,CA). IHC was performed using an automated immunostainer (NexES; Ventana Medical Systems, Tucson, AZ). Slides were incubated with primary antibody (Ventana Medical Systems Anti-Prion 99, Ventana Medical Systems, Tucson, AZ) for 32 minutes. The remaining procedure was performed using a commercially available alkaline phosphatase immunostaining technique (Ventana ultraView Universal Alkaline Phosphatase Red Detection Kit, Ventana Medical Systems, Tucson, AZ) according to manufacturer's instructions. Slides were counterstained with Hematoxylin. Positive control tissues, consisting of obex and retropharyngeal lymph node from a scrapie-positive sheep, were prepared with each set of 20 slides.

Immunohistochemically stained tissues were evaluated by 4 pathologists experienced in TSE diagnosis. A test on any single tissue was recorded as positive for scrapie when globular red reaction product within the cytoplasm of tangible body macrophages, or fine

granular red reaction product within a germinal center of a lymphoid follicle or within grey matter was identified within a tissue section. If no precipitate was identified within a section, the test result for that tissue was recorded as negative for scrapie. Results from sections of lymphoid tissue lacking scrapie-specific immunostaining and containing fewer than 6 lymphoid follicles were recorded as "insufficient lymphoid follicles for determination" (ISF). Positive tissues were re-evaluated and confirmed by a second pathologist.

Diagnostic Grade

After approximately 10 months had passed since the start of the study, it became apparent that RAMALT ISF results seemed more common amongst RAMALT biopsies which contained a substantial proportion of mucosa comprised of nonkeratinizing stratified squamous epithelium. Squamous mucosa exists caudal to the recto-anal junction; therefore, such biopsies were taken from an inappropriate area. A grading scheme was devised to help determine the association of caudal biopsy location (as evidenced by the presence of squamous mucosa) with ISF results. Grade 1 was assigned to RAMALT biopsies that contained no squamous mucosa. Grade 2 was assigned to RAMALT biopsies with $< 50\%$ of mucosa comprised of squamous epithelium, and grade 3 was assigned to those with $\geq 50\%$ squamous epithelium.

Classification of Test Results and Scrapie Status

Test results from all tissues were dichotomized into positive or negative results. If the inappropriate subgross location of obex was sampled (i.e. area of medulla oblongata not

containing the dorsal motor nucleus of the vagus nerve), the result was excluded from analysis (i.e. recorded as a missing value). When a lymphoid tissue yielded ISF results, these were considered un-interpretable results and were excluded from test sensitivity analysis (i.e. dichotomized test results were recorded as missing values).

Dichotomous test results were created for interpreting RV and LV RAMALT biopsies in parallel, right and left third eyelid biopsies in parallel, and RV and LV RAMALT and right and left third eyelid biopsies in parallel. For parallel test results, if one or more biopsies tested positive, the result was designated as positive. If all biopsies were not positive, the test in parallel result was negative. Parallel test results were recorded as missing values if one or more of the required tests were not completed or if all required tests had produced ISF results.

A sheep's scrapie status was determined using dichotomized results of the reference ("gold standard") test. The reference test comprised PrP^{res} IHC applied to obex and tonsil and/or retropharyngeal lymph node in parallel. A sheep's status was considered scrapie-confirmed when one or more of these tissues had scrapie-specific immunostaining. A sheep's status was considered scrapie-non-confirmed when all evaluated tissues lacked scrapie-specific immunostaining.

Lymphoid Follicles

For each scrapie-confirmed sheep, the total number of lymphoid follicles, and the number of lymphoid follicles containing scrapie-specific immunostaining were counted in each

RAMALT and third eyelid biopsy. A lymphoid follicle was defined as a discrete or expansile aggregate of mononuclear cells. The lymphoid follicles in right and left eyelid biopsies were totaled.

Statistical Analysis

Descriptive and analytical statistics were performed using commercially available software (SAS 9.1, SAS Institute Inc., Cary, NC). For all analyses, $p < 0.05$ was regarded as significant.

Sheep included in statistical analysis were those that had complete verification of scrapie status using the reference ("gold standard") test. Complete verification required a test result for obex in addition to either retropharyngeal lymph node or palatine tonsil; sheep with incomplete verification were those lacking either obex or both retropharyngeal lymph node and palatine tonsil.

Sheep were classified into breed groups according to face color (Table 1). Cross-bred sheep were recorded as the breed that was considered most representative; when features of a breed did not predominate or were not recognized, cross-bred sheep were classified by face color and recorded as cross-bred sheep.

For continuous variables, such as age and follicle number, normality of data was evaluated using an Anderson-Darling normality test and normal probability plot. A Wilcoxon rank-sum test was used to evaluate the difference in median age between

scrapie status groups and test result groups. Sensitivity was calculated as the percentage of scrapie-confirmed sheep that had positive test results. Exact 95% confidence limits were estimated using a binomial distribution. Scrapie-confirmed sheep were cross-classified by results of 2 tests; the McNemar Chi-Square test was used to test for differences in sensitivity between 2 biopsy sites. The Pearson Chi-Square test was used to test for differences in the proportion of positive RAMALT results between breed class groups (black-face, white-face, other), age groups (< 2 years, 2-5 years, and > 5 years), and PNRP genotype at codon 136 groups (AA_{136} and not-AA_{136}). The kappa statistic was used to determine the level of agreement between 2 tests that exceeded the level expected by chance.

The Pearson Chi-Square test was used to test for association of diagnostic grade and age group (< 2 years or ≥ 2 years) with dichotomized test results (ISF versus non-ISF results). The McNemar Chi-Square test was used to test for the difference in proportion of ISF results between biopsy sites. The difference in median total lymphoid follicle numbers per biopsy between biopsy sites was evaluated using a Wilcoxon signed rank test. The difference in median total follicle numbers per biopsy between age groups was tested using a Wilcoxon rank-sum test.

Using total follicle number and number of follicles containing scrapie-specific immunostaining, a logistic model was constructed to predict the probability (PF) of a lymphoid follicle having scrapie-specific immunostaining occurring in RAMALT and third eyelid biopsies from scrapie-confirmed sheep. Wald 95% confidence limits were

constructed for these estimates. The lower bounds of the 95% confidence limits were used to estimate the probability of no lymphoid follicles being positive in a scrapie-confirmed sheep, given *n* follicles were evaluated ($p = 1 - (1 - PF)^n$, where n = the number of follicles evaluated).

Results

Animals

543 sheep were enrolled in the study from November, 2006 to October, 2007. 532 sheep were included in analysis; of the 11 not included: 4 had incomplete verification using reference test, and 7 were sheep that could not be confirmed as meeting criteria for enrollment. For the purpose of this report, sheep included in analysis will be referred to as "the study population".

The study population comprised 497 females and 35 males, and represented 11 breeds and 8 different PNRP genotypes (Table 5.1). Samples were collected by approximately 38 veterinarians or veterinary technicians and were obtained from 60 flocks of 22 states. 524 sheep of the study population were high-risk sheep from scrapie-exposed flocks, while 8 were sheep with clinical signs consistent with scrapie.

90 sheep of the study population were designated as scrapie-confirmed by the reference test (prevalence = 16.9%). Scrapie-confirmed sheep were derived from 16 flocks. The median (range) number of scrapie-confirmed sheep obtained per flock was 2 (1-29). 89.4% of scrapie-confirmed sheep with known PNRP genotype were AA_{136}, and 10.6%

were AV_{136}. All but one scrapie-confirmed sheep with known PNRP genotype (98.9%) were QQ_{171}; the remaining sheep was RR_{171}. Three of 8 sheep with scrapie-consistent clinical signs were scrapie-confirmed.

The median (range) age of sheep was 3.5 (1-11.0) for the study population as a whole and 3.0 (1.0-10.0) for scrapie-confirmed sheep. The proportion of scrapie-confirmed sheep varied among age groups ($p < 0.05$), and the highest proportion of scrapie-confirmed sheep was within the 2-5 year age group (Table 5.1).

Complications

352 (66.3%) sheep had the RV and/or LV RAMALT biopsies sampled on the same day as postmortem collection of LD RAMALT biopsies, precluding observation of potential complications associated with the biopsy procedure. The median (range) of time interval between antemortem and postmortem sampling was 0 (0-28) days.

Complications potentially associated with the RAMALT biopsy procedure were reported by veterinarians for 3 sheep. One sheep developed a rectal prolapse following biopsy collection. Substantial rectal hemorrhage was noted for 1 sheep, and a biopsy perforated the rectum of 1 sheep.

Sensitivity estimates

Sensitivity estimates for RAMALT and third eyelid biopsies, and contingency tables summarizing dichotomized test results as compared to scrapie status determined by the

reference test are provided in Table 5.2. Sensitivity estimates for RV and LV RAMALT biopsies were stratified by breed class, age group, and PNRP genotype at codon 136 (Table 5.3). There was no statistically significant difference between RV RAMALT biopsy and LV RAMALT biopsy sensitivity ($p = 0.65$, power = 0.01), or between RV RAMALT biopsy and postmortem LD RAMALT sample sensitivity ($p = 0.41$, power = 0.03). The agreement of results from RV and LV RAMALT biopsies was very high (kappa = 0.95).

Forty-five scrapie-confirmed sheep had RV and LV RAMALT biopsies and right and left third eyelid biopsies tested, allowing comparisons between the sensitivity of these two tests. Of these sheep, there were 7 that were positive by RV and LV RAMALT biopsies in parallel, and negative by right and left third eyelid biopsies in parallel. In contrast, there were 2 that were positive by right and left third eyelid biopsies in parallel, and negative by the RV and LV RAMALT biopsies in parallel. Although the agreement of right and left third eyelid biopsies in parallel with RV and LV RAMALT biopsies in parallel was very high (kappa = 0.94), there was a statistically significant difference between in the sensitivity of these two tests ($p < 0.05$).

False negative results from RV and LV RAMALT biopsies in parallel occurred for 7 of 84 scrapie-confirmed sheep that were tested with both RV and LV RAMALT biopsies. Five of these sheep had negative obex results, 4 were from a very heavily infected flock, and 6 had an interval between rectal biopsy sampling and postmortem collection of tissues for the reference test that was ≥ 5 days (1 of which was positive on the

postmortem collected LD RAMALT biopsy). One of these sheep was obex-positive and negative by all lymphoid tissues tested (3 RAMALT biopsies, retropharyngeal lymph node, and tonsil). This sheep was an 8 year old $AA_{136}RR_{171}$ Suffolk. The microanatomic distribution of immunostaining for PrP^{res} (in a section of obex, immunostaining was limited to trigeminal ganglia),[27] and the distinct glycotype profile of PrP^{res} identified with Western blot[28] (data not shown) were consistent with Nor98-like scrapie.

For RV and LV RAMALT biopsies, there was no association between breed class (black-face, white-face, other) and the proportion of positive RAMALT results. The proportion of positive RAMALT results for RV and LV biopsies was highest in the 2-5 year age group ($p < 0.05$ for each biopsy site). The proportion of positive RAMALT results for RV and LV biopsies was higher in AA_{136} sheep than in non-AA_{136} sheep ($p < 0.05$ for each biopsy site).

Un-interpretable test results

The proportions of ISF results for each lymphoid tissue tested are compared in Table 5.4. The differences in proportion of ISF results between RV RAMALT biopsies versus LV RAMALT biopsies, LV RAMALT versus LD RAMALT biopsies, and RV and LV RAMALT biopsies in parallel versus right and left third eyelid biopsies in parallel were statistically significant ($p < 0.05$ for each). There was no statistically significant difference in the proportion of ISF results between LV RAMALT and right and left third eyelid biopsies in parallel ($p = 0.31$, power = 0.12).

The proportion of ISF results varied among diagnostic grade groups ($p < 0.05$), and ISF results were confined to sheep with diagnostic grade of 3 (Table 5.5).

Total lymphoid follicle number in biopsies from sheep with positive scrapie status varied between tissues (Table 5.4). The difference in median total follicle number between RV RAMALT and LD RAMALT biopsies was statistically significant ($p < 0.01$). There was no statistically significantly difference in median total follicle number between RV and LV RAMALT biopsies ($p = 0.75$) or between LV RAMALT or RV RAMALT and right and left third eyelid biopsies ($p = 0.89$ and 0.73, respectively).

For all RAMALT biopsies, the proportion of ISF results was greater for sheep ≥ 2 years of age relative to sheep < 2 years of age ($p < 0.05$). For RV and LV RAMALT biopsies, median age was higher in sheep with ISF results relative to those without (for both sites, sheep with ISF results median = 4.0, for sheep without ISF results median = 3.0; $p < 0.05$). Median age was also higher in sheep that had right and left third eyelid biopsies with ISF results versus those without (sheep with ISF results median age = 3.5, for sheep without ISF results median age = 2.0; $p < 0.05$).

The median total follicle number in RAMALT and third eyelid biopsies was higher in sheep < 2 years of age than in sheep ≥ 2 years of age, but this difference was only statistically significant for RV (median < 2 years age = 39, median ≥ 2 years = 19; $p < 0.05$) and LD RAMALT biopsies (median < 2 years age = 58.5, median ≥ 2 years = 22; $p < 0.05$), but not for LV (median < 2 years age = 32, median ≥ 2 years = 22; $p = 0.09$) or

right and left third eyelid biopsies combined (median < 2 years age = 29, median ≥ 2 years = 16; p = 0.07).

The probability of at least one lymphoid follicle having scrapie-specific immunostaining in an RAMALT biopsy from a scrapie-confirmed sheep, when *n* follicles are evaluated, was estimated for each RAMALT biopsy site using the lower confidents limits for PF values presented in Table 5.4 (Fig. 5.1). To identify 99.9% of scrapie-confirmed sheep, RAMALT biopsies appear to require 7-10 lymphoid follicles. Assuming that the probability of a follicle having scrapie-specific immunoreactivity in an RAMALT biopsy from a sheep with scrapie is no lower than 0.54 (lowest lower confidence limits for RAMALT biopsies in Table 5.4), using a binomial distribution, the probability that no follicle will have scrapie-specific immunostaining if 9 follicles are evaluated is < 0.001. However, 12 scrapie-confirmed sheep of the study population that had 9 or more follicles evaluated per RAMALT biopsy had no follicles containing scrapie-specific immunostaining identified in at least 1 RAMALT biopsy (7 were lacking positive-staining follicles in the RV RAMALT biopsy, 9 lacking positive follicles in the LV RAMALT biopsy, and 6 lacking positive follicles in the LD RAMALT biopsy). All 12 of these sheep also had at least one other negative lymphoid tissue test and 8/12 were obex-negative. The age of these sheep ranged from 1.3-8; lack of positive-staining follicles when > 9 follicles were evaluated was not limited to young sheep. The median (range) of total follicles evaluated per biopsy from these sheep were 19 (13-36) for the RV, 25 (16-69) for the LV, and 31 (15-43) for the LD RAMALT biopsy.

Discussion

RAMALT is a convenient and safely-collected tissue to test for PrP^{res}. Complications associated with RAMALT sampling are rare and could probably be prevented with training and experience. To reduce rectal hemorrhage, sheep should be kept as calm as possible during and following the biopsy procedure. To prevent rectal perforation, veterinarians and technicians should stay as superficial as possible while undermining mucosa during the biopsy procedure; doing so will also reduce post-biopsy rectal hemorrhage. The biopsy should consist of mucosa only and there should be no connective tissue attached to the underside of the biopsy.

PrP^{res} IHC applied to RAMALT biopsies is a fairly sensitive method for diagnosing preclinical scrapie in live sheep. A study evaluating PrP^{res} IHC applied to RAMALT samples collected postmortem from experimentally and naturally-infected sheep estimated sensitivity to range from 86.0-97.1% (relative to PrP^{res} IHC applied to obex, retropharyngeal lymph node, tonsil, and ileum in parallel), depending on presence or absence of clinical signs.[23] The present study estimates sensitivity of PrP^{res} IHC applied to antemortem-sampled RAMALT to range from 89.3-92.9%. The higher sensitivity estimates might be attributed at least in part to the distinctive embedding technique used by the present study where the mucosal surface was embedded flush with the plane of tissue sectioning such that the number of lymphoid follicles included per tissue section was maximized. No difference in sensitivity was detected between 3 RAMALT sites; however, power for these comparisons was very low given the limited sample size of scrapie-confirmed sheep in the study population.

The false negative rate (1 – sensitivity) for RV and LV RAMALT biopsies tested in parallel was 8.3%. One false negative occurred as a result of a scrapie-confirmed sheep having Nor98-like scrapie, the most frequently recognized form of "atypical" scrapie, which does not accumulate PrP^{res} in lymphoid tissues and is thought to be caused by a novel strain of scrapie or represent a sporadic disease similar to Creutzfeldt-Jakob disease in humans.[27] Since many of the sheep with false negative results were obex-negative and/or from heavily infected flocks, it is likely that these sheep were in an early stage of disease where PrP^{res} may not had reached detectable levels in RAMALT. Since 6 sheep with false negative results had an interval between collection of tissues for RAMALT testing and collection of tissues for reference testing that was ≥ 5 days (1 of which was positive by postmortem-collected LD RAMALT biopsy), it is possible that some of the false negatives could have been attributed to PrP^{res} reaching detectable levels during the sampling interval. Although this occurrence seems unlikely given the short time-span, allowing a period of time to elapse between RAMALT and third eyelid biopsy sampling and postmortem sampling of tissues for the reference test may have created downward bias in the present study's sensitivity estimates for antemortem tests.

The sensitivity of RAMALT PrP^{res} IHC appears to meet or exceed the sensitivity of PrP^{res} IHC applied to third eyelid. The sensitivity of PrP^{res} IHC applied to a single third eyelid biopsy collected from a live subclinical sheep has been estimated to be 72.5%.[22] This study used PrP^{res} applied to obex as the reference test. Since lymphoreticular tissue accumulate PrP^{res} at an earlier stage of disease than does central nervous system tissue,[29]

excluding retropharyngeal and/or tonsil from the reference test may have inflated this sensitivity estimate. Nonetheless, the present study found the sensitivity of PrP^{res} IHC applied to a single RAMALT biopsy was similar to the sensitivity of PrP^{res} IHC applied to two (right and left) third eyelid biopsies, and the sensitivity of PrP^{res} IHC applied to two RAMALT biopsies was significantly higher than that of two third eyelid biopsies (91.7 vs. 86.1%).

Host factors that are associated with susceptibility to disease, progression of disease, or expression of biomarkers for disease, may provide the basis for variation in test performance between subpopulations of animals.[30] Tests sensitivity may be lower in younger animals that have been infected for a shorter period of time and have therefore had less time to accumulate detectable levels of PrP^{res} in any tissue. Although the proportion of positive RAMALT biopsy results varies between age groups, no consistent trends in the magnitude of difference of RV and LV RAMALT biopsy sensitivities between age group stratums were observed in the present study. Test sensitivity may be higher in sheep with clinical signs that are in a late stage of infection; however, stratums for presence of absence of scrapie-consistent clinical signs could not be evaluated since very few scrapie-confirmed sheep had clinical disease (n = 3). Test sensitivity may be higher in sheep with certain PNRP genotypes that are associated with greater susceptibility for disease. Susceptibility to scrapie is most strongly associated with QQ_{171} genotype;[31,32] however, since all but 1 scrapie-confirmed sheep of the study population were QQ_{171}, the effect of this genotype on test sensitivity could not be evaluated. In the U.S., most cases of scrapie occur in A_{136} sheep. Although the proportion of positive

RAMALT results was higher in AA_{136} sheep, no clear trends were apparent when comparing RV and LV RAMALT sensitivites between AA_{136} sheep and non-AA_{136} sheep. Adult black-faced U.S. sheep are 38 times more likely to test positive for scrapie at slaughter than white-faced U.S. sheep.[14] In the present study, sensitivity estimates for RV and LV RAMALT biopsies were higher in black-faced breeds than in white-faced breeds (a difference that was not statically significant). As the precision of sensitivity estimates were limited by small numbers of scrapie-confirmed animals represented by each stratum, further study is warranted as true sensitivity difference may exist. Overlapping CIs for all stratum-specific sensitivity estimates suggest that there is no statistically significant difference in sensitivity between sheep of different face color, age group, or PNRP genotype at codon 136.

For the purposes of this study, when a single RAMALT or third-eyelid biopsy contained fewer than 6 follicles and lacked scrapie-specific immunostaining (ISF), test results were considered inconclusive since there was too little evaluated tissue to detect disease. When two or more tissues were interpreted in parallel, the in-parallel test result was only considered ISF when all tissues were ISF (i.e. if one or more tissue was ISF, but the other(s) were not, the result of parallel testing was determined by the other tissue(s)). ISF test results were not used when estimating sensitivity or when conducting sensitivity analyses. This approach represents the manner with which test results are interpreted during NSEP-associated field investigations. Sheep with ISF results are not considered negative for scrapie, and repeat biopsy or indemnification is often pursued for sheep with ISF results. If two or more samples are tested, positive or negative results of a single

tissue suffice to determine scrapie status. Those using the RAMALT test should be aware that if ISF results are classified as negative test results, the true RAMALT sensitivity is probably significantly lower than the estimates provided herein.

Since third eyelids contain limited lymphoid tissue, they are not amenable to repeat or multiple biopsy. Therefore, ISF results derived from a third eyelid test present a dilemma when conducting NSEP-relevant flock investigations. If a high-risk sheep cannot be verified with antemortem testing as negative for scrapie, indemnification and postsmortem testing is usually conducted. If indemnification is not elected, while awaiting postmortem testing following natural death or slaughter, unnecessary interventions to prevent the spread of scrapie may be placed on the flock since the flock's true scrapie status remains unknown. The frequency of ISF results from a single eyelid biopsy collected from live sheep by an experienced technician has been estimated at 20% to 38.5%.[21,22] Therefore, to increase the likelihood of obtaining a suitable sample for diagnosis during initial testing, it is often recommended that two eyelid biopsies are collected from each sheep (one from each eye). In the present study, the frequency of obtaining ISF results for both third eyelid biopsies was 22.5%. The frequency of obtaining ISF results from a single RAMALT biopsy ranged from 15.1-32.0%, depending on RAMALT site. The LD position had the least frequency of ISF results; however, this finding is confounded by postmortem sampling in which a sample collector may be more prone to collect a larger sample and/or more likely to correctly sample tissue from the precise anatomical area containing RAMALT. The frequency of 2 simultaneously collected RAMALT biopsies producing ISF results was 14.1%; this was statically

significantly lower than the ISF frequency of 2 third eyelid biopsies. In addition, 2 RAMALT biopsies together contained more lymphoid follicles than 2 eyelids together. Therefore, RAMALT biopsies appear to be less likely to produce ISF results than third eyelid biopsies. Furthermore, should ISF results be obtained, RAMALT is much more abundant than third eyelid lymphoid tissue and can be sampled repeatedly.

The frequency of ISF results for RAMALT biopsies (15-32%) may have been biased by the inexperience of veterinarians and technicians at performing the RAMALT biopsy technique. ISF results were much more likely to be assigned to biopsies that contained substantial squamous mucosa, indicating that they had been collected from an area of the recto-anal interface that was caudal to the position of RAMALT. Furthermore, another study which explored the potential for RAMALT PrP^{res} IHC to diagnose scrapie using experimentally infected sheep found 87% of RAMALT sections to contain lymphoid follicles when sampling 2 cm cranial to the rectoanal line.[24] Therefore, to avoid receiving ISF results, inexperienced RAMALT sample collectors should be sure to collect tissue which extends at least 1 cm cranially from the anal mucocutaneous junction. Use of a headlamp, proper animal restraint, topical anesthetic, and rectal speculum may improve visualization of the appropriate anatomical site for sampling.

For all RAMALT biopsies ISF results occurred more frequently amongst sheep ≥ 2 years of age versus sheep < 2 years of age, and for RV and RD RAMALT biopsies, median total follicle number was higher in sheep < 2 years of age relative to sheep ≥ 2 years of age. Therefore, as reported for the third eyelid biopsy,[21] RAMALT biopsies may be most

likely to produce suitable samples for diagnosis in young sheep with presumably robust lymphoid tissue.

The probability of a lymphoid follicle in a RAMALT biopsy from a scrapie-confirmed sheep to contain scrapie-specific immunostaining was used to estimate the likelihood of a biopsy from a sheep with scrapie to not contain scrapie-specific immunostaining give *n* lymphoid follicles were evaluated. To be 99.9% confident that a RAMALT biopsy lacking scrapie-specific immunostaining is not from a sheep with detectable scrapie, it is recommended that a minimum of 9 lymphoid follicles be present for evaluation. Some scrapie-confirmed sheep of the study population had more than 9 follicles evaluated, with none containing scrapie-specicfic immunostaining. Since these sheep were frequently obex-negative and negative by other lymphoid tissues evaluated, they seem to have been in an early stage of infection. The median follicle numbers evaluated per biopsy ranged from 19-24 and ranged as high as 69. Therefore, detecting these sheep might require that an unpractical number of lymphoid follicles be evaluated per biopsy and might not even be possible given the imperfect sensitivity of the RAMALT biopsy.

During the study period, approximately 60% of eligible scrapie-exposed flocks being investigation and managed by the NSEP were enrolled in the study.(personal communication, Dianne Norden) Based on the distributions of age and PNRP genotype, and on scrapie prevalences, the target population has been suitably represented by this study.[33] A sensitive and diagnostic antemortem test for scrapie may be very useful in other populations. Postmortem-collected RAMALT could be sampled by RSSS to detect

scrapie in sheep that are slaughtered using specialized techniques which preclude collecting tissues from the head (i.e. Halal slaughter). There may also be demand for using the RAMALT biopsy for testing sheep at livestock markets, screening non-high-risk sheep from scrapie-exposed flocks, screening high-risk sheep from flocks not known to be scrapie-exposed, or testing sheep enrolled in the flock certification program. However, caution must be used in generalizing the test performance results presented herein to other populations, since the test may perform differently in populations with different prevalence or with differential expression of disease-associated host characteristics.[30]

The frequentist methods for evaluating test performance used herein are based on the assumption that the reference test is 100% sensitive and specific. However, it is known that PrP^{res} accumulation in any tissue does not occur until weeks to months after infection, presenting a substantial time period in which infected animals can not be detected by PrP^{res} IHC. Therefore, IHC applied to obex and palatine tonsil and/or retropharyngeal lymph node is not 100% sensitive; true sensitivity is unknown. Since the sensitivity estimates for tests evaluated by the present study were relative to the reference test, true sensitivity of these tests is expected to be lower than estimated. Along these lines, the sensitivity of tests evaluated by the present study may be overestimated as a result of conditional dependence between PrP^{res} accumulation in each tissue evaluated.[34] Latent-class methods may be capable of producing more valid sensitivity estimates that account for the absence of a true "gold standard" reference test and the effects of conditional dependence between tests.[35,36]

PrP^{res} IHC applied to RAMALT biopsy appears to be a relatively sensitive approach for antemortem scrapie diagnosis in sheep. Sensitivity at least approximates and appears to exceed the only currently available antemortem test (PrP^{res} IHC applied to third eyelid). In contrast to the third eyelid biopsy, sample collection is easy to perform, and when RAMALT is collected from the appropriate location, few inconclusive test results occur. The availability of RAMALT PrP^{res} IHC to scrapie control programs will reduce the need for indemnification of high-risk sheep while investigating and maintaining scrapie-exposed flocks. RAMALT PrP^{res} IHC is expected to improve the success and rapidity of the U.S. National Scrapie Eradication Program, since flock scrapie status can be more expediently established, facilitating the appropriate placement of scrapie-control mitigations. Given the imperfect sensitivity of the test, to have high confidence in negative test results, it is advisable to pursue confirmatory postmortem testing of high-risk sheep following slaughter or natural death.

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Tables and Figures

Table 5.1. Breeds, PNRP genotypes, and age of sheep represented by the study population.

	n (scrapie-confirmed)	Percent of study population	Percent of scrapie-confirmed sheep
Breed Class			
Black-faced			
Suffolk	144 (27)	27.6	30.0
Black-face cross	32 (8)	6.1	8.9
Hampshire	11	2.1	--
Oxford	11 (1)	2.1	1.1
Shropshire	10 (1)	1.9	1.1
Class Total	208	39.9	41.1
White-faced			
White-face cross	94 (32)	18.0	35.5
Dorset	80	15.4	--
Southdown	67 (12)	12.9	13.3
Montadale	16	3.1	--
Cheviot	12	2.3	--
Finnsheep	7	1.3	--
Rambouillet	5	1.0	--
Corriedale	3	0.6	--
Class Total	287	55.1	48.9
Non-classifiable			
Mottled-face cross	22 (8)	4.2	8.9
Undeterminable	3	0.6	--
Brown-face cross	1 (1)	0.2	1.1
Class Total	26	5.0	10.0
Total	**521 (90)***	**100**	**100**
Genotype			
$AA_{136}QQ_{171}$	316 (75)	61.7	84.3
$AV_{136}QQ_{171}$	83 (9)	16.2	10.1
$AA_{136}QR_{171}$	58	11.3	--
$AV_{136}QR_{171}$	27	5.2	--
QQ_{171}†	15 (4)	2.9	4.5
$VV_{136}QQ_{171}$	8	1.6	--
$AA_{136}RR_{171}$	4 (1)	0.8	1.1
$VV_{136}QR_{171}$	1	0.2	--
Total	**512 (89)‡**	**100**	**100**
Age-group			
< 2 years	119 (23)	22.4	25.6
2-5 years	260 (52)	48.9	57.8
> 5 years	153 (15)	28.8	16.6
Total	**532 (90)**	**100**	**100**

*breed information was not available for 11 sheep.
†information regarding the genotype of the 171 PNRP codon was not available.
‡no information regarding PNRP genotype was available for 20 sheep (1 of which was scrapie-confirmed).

Table 5.2. Sensitivity estimates for RAMALT and third eyelid biopsies and summary of test results stratified by designation for scrapie status. Reference test consisted of PrP^{res} IHC applied to obex in addition to retropharyngeal lymph node and/or palatine tonsil in parallel; if any one of these tissues contained scrapie-specific immunostaining, the sheep was designated as scrapie-confirmed.

Test		Scrapie Status		Sensitivity (95% CI)
		Confirmed	Non-Confirmed	
Obex (n = 532)				
	+	59	0	N/A
	-	31	442	
Total		90	442	
Retropharyngeal Lymph Node (n = 530)				
	+	88	0	N/A
	-	2	440	
Total		90	440	
Palatine Tonsil (n = 502)				
	+	77	0	N/A
	-	3	422	
Total		80	422	
RV (n = 362)				
	+	67	0	89.3 (80.1, 95.3)
	-	8	287	
Total		75	287	
LV (n = 392)				
	+	70	0	87.5 (78.2, 93.8)
	-	10	312	
Total		80	312	
LD (n = 446)				
	+	78	0	92.9 (85.1, 97.3)
	-	6	362	
Total		84	362	
EYE (n = 224)				
	+	37	0	86.1 (72.1, 94.7)
	-	6	181	
Total		43	181	
RV + LV in parallel (n = 457)				
	+	77	0	91.7 (83.6, 96.6)
	-	7	373	
Total		84	373	
RV + LV + EYE in parallel (n = 274)				
	+	44	0	97.8 (88.2, 99.9)
	-	1	229	
Total		45	229	

n = number of tested samples with positive or negative results
RV = right ventral RAMALT biopsy (collected antemortem)
LV = left ventral RAMALT biopsy (collected antemortem)
LD = left dorsal RAMALT biopsy (collected postmortem)
EYE = right and left third eyelid biopsies (collected antemortem or postmortem) tested in parallel

Table 5.3. Sensitivity estimates for RAMALT biopsies stratified by breed class, age group, and PNRP genotype group. Reference test consisted of PrP^{res} IHC applied to obex in addition to retropharyngeal lymph node and/or palatine tonsil in parallel.

	RV			LV		
	n	Se	95% CI	n	Se	95% CI
Breed						
Black-face	32	93.8	79.2, 99.2	35	91.4	76.9, 98.2
White-face	39	84.6	69.5, 94.1	38	81.6	65.7, 92.3
Non-classifiable	4	100	39.8, 100	7	100	59.0, 100
Age						
< 2 years	23	87.0	66.4, 97.2	23	95.7	78.1, 99.9
2-5 years	42	92.9	80.5, 98.5	44	86.4	72.7, 94.8
> 5 years	10	80.0	44.4, 97.5	13	76.9	46.2, 95.0
Genotype						
AA_{136}	61	90.2	79.8, 96.3	66	86.4	75.7, 94.6
Not AA_{136}	14	85.7	57.2, 98.2	14	92.9	66.1, 99.8

n = number of scrapie-confirmed animals tested per specified group
RV = right ventral RAMALT biopsy (collected antemortem)
LV = left ventral RAMALT biopsy (collected antemortem)

Table 5.4. Comparison of tests for "insufficient lymphoid tissue for determination" (ISF) results, median follicle number, and probability of lymphoid follicles containing scrapie-specific immunostaining (PF).

Tissue tested (n)	Percent ISF Results	Median follicle no.*	PF (95% CI)
RV (532)	32.0	20	0.60 (0.58, 0.62)
LV (532)	26.3	22.5	0.66 (0.64, 0.68)
LD (525)	15.1	27	0.55 (0.53, 0.57)
EYE (289)	22.5	17	--
RV + LV (532)	14.1	--	--
Palatine tonsil (511)	1.7	--	--
Retropharyngeal Lymph Node (530)	0	--	--

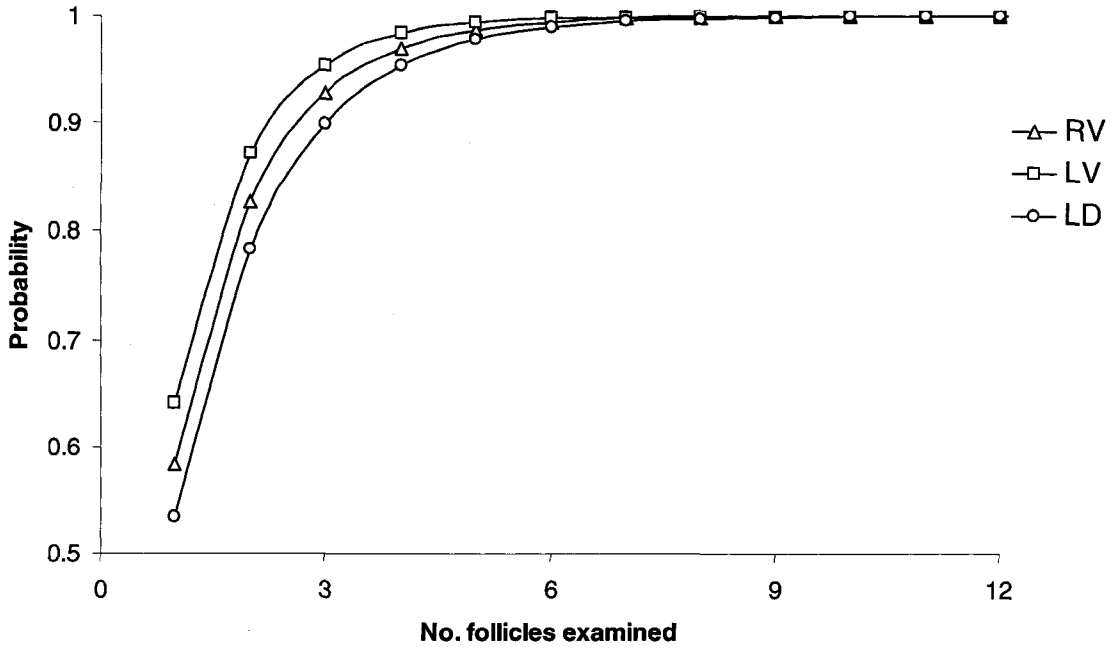
n = number of samples or pairs of samples tested
PF = probability of a lymphoid tissue containing lymphoid follicles with scrapie-specific immunostaining in scrapie-confirmed sheep
RV = right ventral RAMALT biopsy (collected antemortem)
LV = left ventral RAMALT biopsy (collected antemortem)
LD = left dorsal RAMALT biopsy (collected postmortem)
EYE = right and left third eyelid biopsies (both with "ISF" results, collected antemortem) tested in parallel
RV + LV = two RAMALT biopsies (both with "ISF" results, collected antemortem) in parallel
*Median lymphoid follicles per tissue from scrapie-confirmed sheep; for EYE, median represents the median of the sum of follicles in right and left third eyelid biopsies

Table 5.5. Number of sheep with RAMALT biopsies containing an insufficient number of lymphoid follicles (ISF results) stratified by diagnostic grade.

Test Results	No. of Animals					
	Diagnostic Grade by Tissue					
	RV		LV		DV	
	1-2	3	1-2	3	1-2	3
ISF	0	1	0	1	0	3
Pos/Neg	9	1	10	0	9	0

RV = right ventral RAMALT biopsy (collected antemortem)
LV = left ventral RAMALT biopsy (collected antemortem)
LD = left dorsal RAMALT biopsy (collected postmortem)

Figure 5.1. Probability of at least one lymphoid follicle containing scrapie-specific immunostainting as a function of number of follicles examined per lymphoid tissue biopsy from a scrapie-confirmed sheep.



CHAPTER 6

Conclusions and Future Research

Transmissible spongiform encephalopathies continue to hold negative public perception. TSE surveillance programs in the U.S. are conducted to protect animal health through supporting TSE control programs, and to alleviate the impact of the existence of animal TSEs on the U.S. economy. TSE surveillance and control programs are challenged by diagnostic assays which only detect late-stage disease, the lack of practical antemortem tests, the infrequent disease occurrence, the degree to which prions are resistant to inactivation, and incomplete understanding of transmission. Research conducted in the studies herein addressed questions relevant to improving TSE diagnosis and surveillance. These works along with thorough review of TSE surveillance practices in the U.S. have identified several relevant issues that could benefit from further attention and investigation.

BSE surveillance ensured trading partners that the existence of BSE in U.S. cattle was extremely limited. A cycle of surveillance planning, implementation, and evaluation was used for improve BSE surveillance and to adapt to new results-directed objectives. This cycle is an ideal model for surveillance oversight as it facilitates logical and efficient allocation of resources. The production of written structured surveillance plans for BSE surveillance ensured the conduct of a comprehensive study using methods with sound scientific reasoning and merit. Moreover, the documentation of the BSE surveillance plan evidenced the program's credibility and validity and facilitated management of the plan's implementation. Perhaps most importantly, structured surveillance planning and

evaluation together ensured that the BSE surveillance program has been and is fulfilling the needs that called for its establishment.

Through evaluation and planning of BSE surveillance, several impediments that may be common amongst sizeable animal disease surveillance programs in the U.S were recognized. First, there is need to improve coordination between government units designing a surveillance program and units responsible for achieving disease diagnosis such that testing and diagnostic strategy could become more formally integrated into surveillance plans. Diagnosticians would benefit from the knowledgebase of epidemiologists when designing diagnostic strategy. Epidemiologists would benefit from the knowledge-base of diagnosticians regarding test rapidity, cost, and newly available diagnostic techniques. Furthermore, this relationship would direct the allocation of surveillance-related testing activities to laboratories best suited for efficient and reliable diagnosis and would help ensure that laboratory resources are used with maximum efficiency while maintaining surge capacity. Second, since all animal health surveillance programs rely upon voluntary participation of owners or producers, greater effort should be given to enlisting their participation. Efforts could include providing pre-defined, fair consequences for identification of disease in animal property (possibly detailed within written surveillance plans). Provision of incentives or compensation for losses is almost a necessity to encourage involvement. Structured nationwide education of the benefits of detecting or eradicating a disease is needed to teach owners and producers why their involvement in surveillance programs will be beneficial. Furthermore, efforts should be made to direct the mindset of invested production industries; surveillance programs

provide an economic advantage that is funded by the public and should be regarded more as privilege than requirement. Finally, reliable animal identification systems are needed to support surveillance programs. Without identification standards, it is very difficult to trace the movement or origin of animals, precluding surveillance from supporting disease control programs and confounding the description of spatial trends of disease.

Chronic wasting disease surveillance could benefit from the structured model for surveillance planning and evaluation used by this dissertation for BSE. Presently, a single comprehensive national surveillance plan that outlines standards for CWD surveillance practices in farmed and wild cervids does not exist. The inconsistencies between state-based CWD surveillance activities, as a result of deficit in funding and communication between regions, make it very difficult to ensure practices and standards are uniform between states. When states and regions are working independently, it is nearly impossible to manage surveillance-derived data in a manner that allows results to be analyzed on a nation-wide basis; a national CWD surveillance database does not currently exist. Without compiled surveillance methods or data, it is difficult to assemble a holistic perspective of CWD surveillance activities that have taken place in the U.S. over the last decade. It is even more challenging to compile or analyze information derived from these surveillance activities or to determine how their outcomes have been or will be used to impact policy development and agency decision making. Ttherefore, these activities seem to represent disease monitoring, not surveillance. Since CWD is an uncommon disease, perhaps a formal surveillance plan could use targeted sampling methodology, thereby reducing the resources needed to detect disease. This methodology

could involve a points-based system similar to that used by BSE surveillance. However, such surveillance plans would require results from studies that identify host characteristics associated with increased likelihood for disease. CWD is not evenly distributed amongst U.S. cervid populations. A CWD surveillance plan might require risk analyses to help direct geographic-based sampling methods and allocation of surveillance-supporting funds. States without endemic CWD should receive financial support for surveillance only when risk assessment has prioritized need for surveillance in the given area.

Resources should only be allocated to surveillance programs that are capable of meeting their stated objectives. In the context of disease control programs, investments into surveillance should be large enough to provide results that tangibly support the control program. When considering surveillance that is implemented to detect CWD in non-endemic areas, early detection of the rare disease may require considerable investments. If the objective of early detection is to rapidly institute disease control measures, these measures should be known to reduce prevalence. If the available control measures are ineffective, than consumption of public resources for disease surveillance is not justifiable in this context. Research is needed to formally evaluate whether control measures which have been explored for CWD in wild cervids are efficacious.

The diagnostic strategy used by a surveillance program must consider how a test will perform under field conditions. In terms of postmortem samples, sample quality may be affected by the interval between death and sample collection and the interval between

sample collection and testing. Loss of PrP^{res} detectability was demonstrated over time when brain samples from CWD-affected elk were incubated under heated conditions. This seems to imply that animals that are marginally TSE-positive may have false negative test results when PrP^{res} immunoassays are applied to brain tissue that has been subjected to such conditions. Therefore, TSE surveillance programs should consider likelihood of adequate sample quality when selecting subpopulations to target with surveillance, and should interpret negative results from deteriorated samples with caution (especially when samples are derived from clinically suspicious animals).

This study provided evidence that PrP^{res}, a constituent of the infectious prion protein, may be susceptible to degradation and possibly deactivation when using disposal or decontamination systems using high temperatures over time. Future study is needed using bioassay to characterize the effect of heated incubation on infectivity. This research would provide the basis for ultimately accepting this hypothesis. If infectivity is reduced, study should be directed towards evaluating the potential for disposal systems such as mortality composting, which operates at high temperatures, to naturally degrade prions. These studies could concurrently evaluate several microbial factors, such as consortium of species or proteolytic enzymes involved, for contribution to prion degradation.

Surveillance programs must constantly adapt to new scientific information regarding epidemiology, pathogenesis, and recognition of disease. Newly available diagnostic assays must be carefully scrutinized prior to be adopted by a surveillance program. PrP^{res}

immunohistochemistry applied to recto-anal mucosa-associated lymphoid tissue (RAMALT) is a newly available antemortem test for scrapie diagnosis that appears to have sensitivity which approximates currently available antemortem tests. Furthermore, this test is easier to perform than the only other available antemortem test (PrP^{res} IHC applied to third eyelid biopsy), and in contrast, it can be used to test a single animal multiple times. Although this test is expected to improve the efficiency of investigation and management of scrapie-exposed flocks by the National Scrapie Eradication Program, the test may also be useful when evaluating several other populations for scrapie (including goats, slaughtered sheep, high-risk non-exposed sheep, sheep enrolled in flock certification programs, or sheep transiting livestock markets). Since other populations may have characteristics which provide a biological foundation for reducing or enhancing sensitivity of the test, further study is needed to elucidate the effect of certain host characteristics, including age group, PRNP genotype, breed, and clinical signs on test sensitivity. Furthermore, prior to abandoning the third eyelid test, a study which tests enough scrapie-confirmed animals to reach an appropriate level of statistical power in making comparisons between RAMALT and third eyelid sensitivity should be conducted. The sensitivity estimates provided for this test are only relevant in comparison to the elected criterion-referenced standard (PrP^{res} IHC applied to obex and retropharyngeal lymph node and/or palatine tonsil in parallel). Since this standard does not have perfect sensitivity, estimates have been biased. Bayesian analysis which accounts for the absence of a "gold standard" and for conditional dependence between tests could produce a more accurate estimate of sensitivity.

The availability of practical live animal tests, such as PrP^{res} IHC applied to RAMALT biopsy, may reduce the need for depopulation of sheep at risk for scrapie and may bring about earlier detection of disease in newly affected flocks. As an antemortem test is badly needed for CWD diagnosis, and as PrP^{res} has been observed to accumulate in RAMALT of CWD-affected cervids, evaluation of PrP^{res} IHC applied to RAMALT of cervids is warranted.

Several "rapid tests" have emerged in the last decade that use immunodetection of PrP^{res} to identify TSE-affected animals which are completed in a shorter time period than IHC. Test turn-around time, and therefore surveillance-directed action, would be improved for scrapie if rapid tests were enlisted by surveillance. These tests have been evaluated in Europe for use on postmortem sampled tissues. However, research is needed to determine whether rapid tests are accurate when applied to tissues that may be collected from live small ruminants, including RAMALT and third eyelid. Ultimately, development of diagnostic assays which detect a marker for disease other than PrP^{res} may have a much more drastic effect on improving test sensitivity and assays which use readily available body fluids such as urine or blood may have a much more drastic effect on test rapidity and cost.